

Biological Control

a global perspective

Edited by
C. Vincent
M.S. Goettel
& G. Lazarovits



BIOLOGICAL CONTROL

A Global Perspective

Case Studies from Around the World

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Edited by

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Contents

List of Contributors	xi
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INTRODUCTION

1 Adventures in Biocontrol	1
<i>George Lazarovits, Mark S. Goettel and Charles Vincent</i>	

CLASSICAL BIOCONTROL PROGRAMMES

2 Search for Biological Control Agents of Invasive Mediterranean Snails	7
<i>James Coupland and Geoff Baker</i>	
3 Introductions of Parasitoids to Control the Apple Ermine Moth in British Columbia	13
<i>Joan E. Cossentine and Ulrich Kuhlmann</i>	
4 Introductions of Parasitoids to Control the Imported Cabbageworm	20
<i>Roy Van Driesche</i>	
5 Biological Control of the Cassava Green Mite in Africa: Overcoming Challenges to Implementation	28
<i>Steve Yaninek</i>	
6 The Multicoloured Asian Ladybird Beetle: Beneficial or Nuisance Organism?	38
<i>Éric Lucas, Geneviève Labrie, Charles Vincent and Joseph Kovach</i>	

7	Introduction of a Fungus into North America for Control of Gypsy Moth	53
	<i>Ann E. Hajek</i>	
8	Weevils Control Invasive Thistles in Canada	63
	<i>Peter Harris</i>	
9	How Many and What Kind of Agents for the Biological Control of Weeds: a Case Study with Diffuse Knapweed	70
	<i>Judith H. Myers</i>	
10	Why is Biocontrol of Common Ragweed, the Most Allergenic Weed in Eastern Europe, Still Only a Hope?	80
	<i>Levente Kiss</i>	
11	Biocontrol for Everyman: Public Participation in a Weed Project	92
	<i>Robert N. Wiedenmann, Susan L. Post, Michael R. Jeffords and David J. Voegtlin</i>	
 INUNDATIVE (OR AUGMENTATIVE) BIOCONTROL PROGRAMMES		
1)	Using Macroorganisms	
12	Biological Control for Insect Pests in Greenhouses: an Unexpected Success	105
	<i>Joop C. van Lenteren</i>	
13	From Chemical to Biological Control in Canadian Greenhouse Crops	118
	<i>Les Shipp, Don Elliott, Dave Gillespie and Jacques Brodeur</i>	
14	An Endemic Omnivorous Predator for Control of Greenhouse Pests	128
	<i>Dave Gillespie, Rob McGregor, Juan A. Sanchez, Sherah VanLaerhoven, Don Quiring, Bernie Roitberg, Robert Footitt, Michael Schwartz and Les Shipp</i>	
15	Entomopathogenic Nematodes: from Science to Commercial Use	136
	<i>Ralf-Udo Ehlers</i>	
16	A Novel Nematode for Management of Slugs	152
	<i>Michael Wilson</i>	

2) Using Microorganisms

Bacteria

- 17 A Novel Bacterium for Control of Grass Grub** 160
Trevor A. Jackson
- 18 How Early Discoveries about *Bacillus thuringiensis* Prejudiced Subsequent Research and Use** 169
Jean-Charles Côté
- 19 Development of Resistance to the Biopesticide *Bacillus thuringiensis kurstaki*** 179
Alida F. Janmaat

Fungi

- 20 How Much Biocontrol is Enough?** 185
Alison Stewart, Kristin McLean and John Hunt
- 21 Control of Root Diseases with *Trichoderma* spp. in Forest Nurseries of Central Siberia** 197
Tatyana I. Gromovych, Valeria A. Tyulpanova, Vera S. Sadykova and Alexander L. Malinovsky
- 22 Commercial Development of *Trichoderma virens* for Damping-off Disease** 203
Robert D. Lumsden and James F. Knauss
- 23 *Trichoderma stromaticum* for Management of Witches' Broom of Cacao in Brazil** 210
Alan W.V. Pomella, Jorge T. De Souza, Givaldo R. Niella, Roy P. Bateman, Prakash K. Hebbar, Leandro L. Loguercio and Robert D. Lumsden
- 24 Lessons Learned from *Sporidesmium*, a Fungal Agent for Control of Sclerotia-forming Fungal Pathogens** 218
Deborah R. Fravel
- 25 Sporodex[®], Fungal Biocontrol for Powdery Mildew in Greenhouse Crops** 224
William R. Jarvis, James A. Traquair and Richard R. Bélanger
- 26 Potential and Limitations of *Microsphaeropsis ochraceae*, an Agent for Biosanitation of Apple Scab** 234
Odile Carisse, Greg Holloway and Mary Leggett

27 Competitive Exclusion of Aflatoxin Producers: Farmer-driven Research and Development	241
<i>Peter J. Cotty, Larry Antilla and Phillip J. Wakelyn</i>	
28 Aflatoxin Control in Cotton and Groundnuts: Regulatory Aspects	254
<i>Shanaz Bacchus</i>	
29 Postharvest Biocontrol: New Concepts and Applications	26
<i>Michael Wisniewski, Charles Wilson, Samir Droby, Edo Chalutz, Ahmed El Ghaouth and Clauzell Stevens</i>	
30 Development of the Mycoherbicide, BioMal®	274
<i>Susan M. Boyetchko, Karen L. Bailey, Russell K. Hynes and Gary Peng</i>	
31 Development of <i>Chondrostereum purpureum</i> as a Mycoherbicide for Deciduous Brush Control	284
<i>William Hintz</i>	
32 Developing the Production System for <i>Chondrostereum purpureum</i>	29
<i>Paul Y. de la Bastide and William E. Hintz</i>	
33 <i>Beauveria bassiana</i> for Pine Caterpillar Management in the People's Republic of China	300
<i>Zengzhi Li</i>	
34 Green Muscle™, a Fungal Biopesticide for Control of Grasshoppers and Locusts in Africa	311
<i>Jürgen Langewald and Christiaan Kooyman</i>	
35 Pollinators as Vectors of Biocontrol Agents – the B52 Story	319
<i>Peter Kevan, John Sutton and Les Shipp</i>	
36 Genetic Modification for Improvement of Virulence of <i>Metarhizium anisopliae</i> as a Microbial Insecticide	328
<i>Raymond J. St. Leger</i>	
Viruses	
37 Madex® and Virosoft^{CP4}®, Viral Biopesticides for Codling Moth Control	336
<i>Charles Vincent, Martin Andermatt and José Valéro</i>	

38 A Nucleopolyhedrovirus for Control of the Velvetbean Caterpillar in Brazilian Soybeans	344
<i>Flávio Moscardi</i>	
39 Abietiv™ a Viral Biopesticide for Control of the Balsam Fir Sawfly	353
<i>Christopher J. Lucarotti, Gaétan Moreau and Edward G. Kettela</i>	
40 Field Tests in the UK of a Genetically Modified Baculovirus	362
<i>Jenny S. Cory</i>	
CONSERVATION BIOCONTROL PROGRAMMES	
41 Control of Mites in Pome Fruit by Inoculation and Conservation	374
<i>Noubar J. Bostanian and Jacques Lasnier</i>	
42 Management of Aphid Populations in Cotton through Conservation: Delaying Insecticide Spraying has its Benefits	383
<i>Don Steinkraus</i>	
43 Management of Pests and Diseases in New Zealand and Australian Vineyards	392
<i>Geoff M. Gurr, Samantha L. Scarratt, Marco Jacometti and Steve D. Wratten</i>	
44 Take-all Decline: Model System in the Science of Biological Control and Clue to the Success of Intensive Cropping	399
<i>R. James Cook</i>	
NETWORKING IN BIOCONTROL	
45 The Biocontrol Network: a Canadian Example of the Importance of Networking	415
<i>Jean-Louis Schwartz, Wayne Campbell and Raynald Laprade</i>	
Index	428

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Adventures in Biocontrol

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Marco Polo, Christopher Columbus and Captain James Cook were explorers who left their mark in history, not only because they travelled into unknown territories but also because they returned home to write about their fascinating adventures. They faced many obstacles and not infrequently suffered from lack of support. Despite all encumbrances, they laboured on because they believed that what they were doing was important and potentially rewarding. Their stubbornness and perseverance led them to great discoveries, if not always the rewards. Likewise, in this book we bring to you a collection of 44 'adventures', each written by scientists who journeyed into the unknown domains of biological control. They made their way towards a goal and, in doing so, faced numerous and unexpected hurdles, which had to be addressed for them to complete their objectives. The field of biological control encompasses not only biology but, as we will learn, also dozens of other disciplines and human activities, including technology, art, business, psychology, economics, law, international trade, sociology and many more. The chapters presented here illustrate that one needs to master combinations of all these elements in order to deploy a successful biological control programme.

Biological Control in a Nutshell

Before we delve into the adventures, we must first ask 'what is biological control?' The idea of biological control is simple: manage a pest by deliberate use of living organisms. In natural ecosystems, such events occur innumerable times and are a major component by which populations of an organism are regulated. In application to agriculture, the goal is to effectively manage populations of beneficial organisms and their ability to reduce the pests' activities within environmental, legal and economic constraints. This is much easier said than done because the constraints can be formidable.

Although the idea of biological control may be simple, definition of the term 'biological control' is not. Scientists can argue about definitions for ever. We will avoid doing so here and let you choose from among the many presented in the following chapters. Entomologists started with the concept that biological control was the use of living organisms (natural enemies) to manage a pest population. Those working in other fields of pest management, however, find this concept difficult to work with and they provide additional concepts that expand the scope of activities that biological control may involve. Wisniewski *et al.*, for instance, (Chapter 29) suggest that for plant pathologists, the entomologist's definition does not work. These authors consider that a plant disease is not an 'organism' but a process. Thus, they define biological control of a plant disease as 'control of a plant disease by a biological process or the product of a biological process.' In Chapter 44, Cook also offers interesting arguments on the need to expand the traditional definition of biological control to include management of plant diseases through manipulation of host ecology and its resistance to pathogens.

We have divided the book according to the three broad categories of biological control, i.e. Classical, Inundative (or Augmentative), and Conservation. In Classical Biological Control, a living organism is introduced to an area where it had not previously existed. The aim is to establish this organism, a natural enemy or competitor, in its new location in order to provide long-term control of a pest. The target pests are, in many cases, non-indigenous to the ecosystem in the first place. In Inundative or Augmentative Biological Control, the aim is to introduce sufficient numbers of the biological control organism(s) to reduce the pest population, at least temporarily. Such introductions would normally need to be repeated, in much the same manner as a traditional pesticide. Conservation Biological Control encompasses efforts to conserve or enrich the biological control agents that are already present, through either manipulation of the environment or crop and pest management practices.

Biological Control: Facing Reality

Those of us who have been working in the area of biological control for many years probably feel humbled by the complexities of the ecosystems we are attempting to impact. In the following chapters you will find examples from around the world as to why this is. These stories reveal the adventures that scientists experienced, starting from the initial search for suitable control agents (e.g. Chapter 2), to their release and introduction to the destined ecosystems, and to the outcomes that in some cases resulted in untold savings from damage caused by insects (e.g. Chapters 3 and 4), pathogens (e.g. Chapters 20 and 27) and noxious weeds (e.g. Chapters 8, 9 and 11). In some cases, these efforts literally saved the staple food supply of several countries (Chapter 5) or a crop vital to the economic survival of growers in a region (Chapter 23). In some cases, the introduction of the control agent was accidental or mysterious (e.g. Chapter 7), while other efforts prospered only after the public became involved in the dispersal of the control agent (Chapter 11). We also see cases where the work remains incomplete and the objectives are still only a hope (Chapter 10). There are situations

where the control agent's behaviour was not as expected and it in itself became a pest (Chapter 6). Nevertheless, Classical Biological Control is a proven powerful management tool, which can provide great benefits if practised cautiously. This example of detrimental effects by an introduced predator occurred at a time when release of biological control agents was not as strictly regulated as it is today. Chapter 6, however, serves as an illustration of why we need to be cautious.

Inundative Biological Control has also seen many successes. For instance, in greenhouses, pest management through biological control has become the foundation of integrated pest management programmes (Chapters 12, 13 and 14). This was brought about because there was a desperate need to control pests that rapidly developed resistance to chemical pesticides within closed ecosystems. In addition, introduction of bees as pollinators greatly increased yields of greenhouse-grown plants but bees proved to be highly sensitive to pesticides. This provided a huge incentive to growers to shift to biological-managed systems where bees were not harmed. This also prompted the search for novel control products, such as entomopathogenic nematodes, products that would have probably been discounted as potential control agents by most plant protection experts (Chapter 15). Such agents, however, are now also providing a means to control slugs (Chapter 16).

Microbial agents are often referred to as 'biopesticides' and have been very successful in the field of biological control. The market for biopesticides, however, is still mainly represented by *Bacillus thuringiensis* (*Bt*) and even it is less than 1% of the gross pesticide sales globally. Is it possible that the very success of this bacterial group as a biopesticide has misdirected our efforts at searching for other uses of this group? One of our authors makes a convincing case that this may be so (Chapter 18). We were also very sure at one time that resistance to biopesticides was not something that could happen easily. Here again, nature teaches us an important lesson: that pests can develop resistance, including biopesticides such as *Bt* (Chapter 19) and baculoviruses (Chapter 37).

Researchers of biological control, and those outside it, often doubt the feasibility for wide-scale use of this technology in today's intensive agricultural and forestry production systems. However, we are told here that insect viruses, once considered as having no possibility for commercialization because of the public's perception that 'viruses' were too dangerous, are now commercially available and applied to tens of thousands of hectares of orchards and forests (Chapters 37, 38 and 39). Many microorganisms are coming to market as commercial products for managing soil-borne diseases of trees (Chapter 21) and agricultural crops (e.g. Chapters 20, 22 and 24), and for control of foliar plant diseases such as powdery mildew (Chapter 25) and apple scab (Chapter 26). The exploitation of plant pathogenic microorganisms for weed control has had several stumbles (Chapter 30) but is now successfully deployed for use in the management of deciduous brush (Chapter 31). Novel production and application methods have been developed to allow more products to reach market, and these are illustrated by articles on the mass fermentation of *Chondrostereum purpureum* (Chapter 32), the use of pollinators to disseminate microorganisms with biological control activity to plants (Chapter 35), and use

of rocket-propelled mortars to better distribute spores of *Beauveria* over the forest canopy (Chapter 33).

There are many plant diseases where control by chemicals was never an option, such as the control of mycotoxin contamination of diverse crops by *Aspergillus* species (Chapter 27). However, application of atoxigenic strains at a few kilograms per hectare protected plants from colonization by toxin-producing isolates. Chapter 28 brings insights into how the regulatory bodies came to evaluate and register the release of these unique products for wide-scale agricultural use. Witches' broom of cacao, a widespread disease in Brazil and tropical South American countries, is being controlled by a parasite of the fungus that incites the disease (Chapter 23). In such areas, chemical spraying would be much too expensive. In both examples, biologicals cost \$5–10/ha, disproving the widely held notion that such treatments are too expensive when compared to chemicals. Often when chemicals are less expensive than biologicals, their potential non-target impacts are rarely factored into the real costs of use.

We find new hope for developing more effective products as our ability to genetically modify biological control agents improves. For instance, transgenic microorganisms can provide more rapid kill times (Chapters 36 and 40). But, as with introductions of generalist predatory ladybeetles, genetic modifications can produce unexpected results (Chapter 36). The search for new means to improve biological control agents and for new agents is potentially a signal of a renaissance for biological control technology. Discovery of a novel bacterium that was commercially developed to control grass grubs in New Zealand (Chapter 17) suggests that biological pest control is alive and thriving.

Biological control successes are almost always associated with the tenacity, communication, team-building ability, and inventiveness of a principal investigator. These investigators invariably have had long-term support from grower groups and enlightened administrators. Many of the products reaching market did so because long-term funding was provided by governments or grower groups, and occasionally by small companies. For the most part, the multinational companies have stayed away from biocontrol products. Yet there is continuing pressure to attract the large companies, with the primary objective being to bring in royalties from commercialization. However, several examples demonstrate that this paradigm is just not working as there is not enough money in such products to lure big companies into this market (e.g. Chapters 15 and 39). Most products actually start as cottage industries; the first preparations of a viral biopesticide now known as Madex[®] were prepared in the bathtub of a student's dorm (Chapter 37). Others are family-run operations, where the motivation for continuation is not primarily the immediate return of the investment but a passion to succeed and bring forth a new biocontrol agent to market (e.g. Chapter 15). Very likely we would have a lot more successes and products on the market if funding agencies and research organizations justified the money spent on such research as a way of improving the environment, as well as providing an alternative pest control strategy to producers. Products such as *Rhizobium* inoculants have been marketed for over half a century, yet few, if any, are protected by patents. They are sold for less than \$10/ha and their benefits on a global scale add up to billions.

Conservation Biocontrol is probably the oldest approach to biological control but, in the modern sense, is also an under-explored realm. Increasing the presence of fungi or bacteria may reduce the activity of a pest by competition or by inducing resistance mechanisms in the host. In such cases, reductions in disease or pest damage occur without a change in the pest's population (Chapter 29). Fungi that can kill aphids reduce not only the damage these pests cause but can also eliminate the need for pesticide application (Chapter 42). Management practices have been developed to conserve beneficial organisms in orchards (Chapter 41) and vineyards (Chapter 43). These examples provide evidence to demonstrate that Conservation Biocontrol has much potential but has been little explored. The ultimate objective in plant protection is to create an environment where pests or pathogens are held in check by competitors or by natural enemies that are already in the environment. Bringing about the balances that create such community relationships and maintaining them such that crop losses are kept below injury thresholds has been most difficult in soil. We have a superb example to show that even here one can tip the equilibrium toward the control agents, and when this occurs long-term disease suppressive conditions are created (Chapter 44). Interestingly, this is achieved by planting the same crop year after year, a practice rarely, if ever, recommended to growers. One may then ask why funding agencies have made such a modest effort globally to develop strategies toward this objective. Cook's chapter not only challenges the prevailing concepts used for controlling plant pathogens but also provides hope that through a better understanding of the ecosystem and the key players that suppress pathogens' activities, we can keep plants healthy at little cost to the grower or to the environment.

Finally, in our last chapter (Chapter 45), we present the adventures experienced in the formation of a Biocontrol Network. It is because of this Network that the idea of this book was born, and many of the chapters are by Network Researchers. We are grateful to the Natural Sciences and Engineering Research Council of Canada and to the Network for financing the creation of this book. This is yet another example of the importance of providing scientists with the opportunities to share resources and ideas in a team effort atmosphere.

The originality of our book is that it showcases clear examples that biocontrol is widely used globally with great success in diverse agro-ecosystems. It is possible that biological control has been oversold for the sake of funding and we as biological control researchers have become deluded by our own rhetoric. The chapters in this book, however, provide convincing arguments that such a view is mistaken. Biological control on a global perspective is a great success.

About Choice of Chapters and Format

We asked authors to explore the positives, impediments and deterrents in getting biological control implemented or in bringing products to market. We wanted the chapters to reflect personal experiences and to include not only the science being pursued but also the mindset and the social environment of the researcher. We believe that these chapters will be a highly valuable resource for teachers,

researchers, and students who wish to experience the historical perspectives and approaches used in the development of biological control. Science managers and regulators will find excellent guidance as to how to help and foster researchers in their efforts to implement biological control or bring products to market.

We are very grateful to all the authors who contributed to this book for so willingly sharing with us their knowledge and life lessons. We have captured only a few examples of the many efforts that exist in the field of biological control and hope that other experiences that have not made it into this edition can be included in future versions.

The publishers presented us with a strict page limit for this book. In order to provide the maximum number of stories, we had to strictly limit the size of each contribution. Although in the past books were an important source of pertinent literature citations, we felt that in this age of the Internet readers now have very easy access to the literature through excellent search engines such as Google Scholar. Consequently, we asked authors to limit the references to about 20 citations. It turned out that this was not an easy task, as scientists obviously feel guilty about offending discoverers of knowledge they are providing. After much persuasion, we were able to more or less meet our objective, although this was not always possible. We offer our apologies to those readers who may feel that their papers should have been cited. The responsibility lies with us and not the authors.

It is our hope that this book will inspire future generations of biocontrol researchers to start their own adventures in biocontrol and make this a much more widely used tool for those who produce food, fibre and energy, which all our lives depend on.

2

Search for Biological Control Agents of Invasive Mediterranean Snails

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Overview: Molluscs are the worst agricultural invertebrate pest after insects, with slugs attacking grain and horticultural crops across the world and snails causing large losses in rice culture and citrus farms. Four species of introduced Mediterranean snails have become serious pests in Australia. This chapter describes the trials and tribulations in the search for parasites which could be used as classical biological control agents for these invasive pests.

Introduction

In Australia, four introduced species of Mediterranean snails, *Theba pisana* (Helicidae), and *Cernuella virgata*, *Cochlicella acuta* and *Cochlicella barbara* (Hygromiidae) have become serious agricultural pests in South and Western Australia and are an increasing problem in western Victoria and southern New South Wales (Baker, 1986, 2002). They cause severe damage and occasionally total destruction to legume-based pastures (e.g. annual medics, lucerne, clovers) and seedling crops (e.g. wheat and barley). Re-establishment of pastures in snail-infested areas is particularly difficult, and stock reject pasture and hay which is heavily contaminated with snails and snail slime. These snails are also agriculturally important in southern Australia because of their habit of climbing on to the heads and stalks of cereals, beans, peas and increasingly grapes for the raisin industry in late spring/early summer to aestivate. During harvest they clog machinery and contaminate the crop. The contaminated crop is then either rendered unacceptable or downgraded. Export shipments of barley from South Australia and Western Australia have been rejected overseas, with Australia's reputation for good-quality grain being damaged. Therefore snails pose a serious threat to the export marketing of Australian cereals.

Surveys and Collections: the First Steps in the Initiation of a Classical Biological Control Programme

Between 1990 and 1996, a biological control programme against these introduced pest terrestrial snails was initiated at the CSIRO European laboratory in Montpellier, France, within the snails' native distributions. During this time, large-scale surveys for parasites which could be used as classical biological control agents were made.

Owing to the lack of knowledge of the parasitoids of these snails, a very large and geographically extensive survey was initiated. Snails were collected from over 400 sites in the region of Montpellier, France and from at least 500 sites in Italy, Portugal, Spain and Morocco during 1991–1995. Sites varied between pastureland, crop edges and littoral zones such as sand dune systems. Living and obviously parasitized snails were collected into plastic cages with gauze tops. Flies which emerged from these snails were then collected and identified to species with the help of the British Museum of Natural History. Over 200,000 snails were sampled.

During these surveys, many dipteran parasitoids with potential as biological control agents were discovered. Of these, flies in the families Sciomyzidae and the Sarcophagidae were seen to have the greatest biocontrol potential (Coupland, 1994, 1996; Coupland and Baker, 1994, 1995, 2004; Coupland *et al.*, 1994). In addition, a nematode, *Phasmarhabditis hermaphrodita*, was discovered which had good activity against several pest snail species (Coupland, 1995).

Choosing the Right Candidate: the Need for Knowledge on General Biology, Efficacy and Host Specificity

Sciomyzids have long been known to be associated with snails, though their larval feeding biology was only elucidated fairly recently by Berg (1953). The relationship between many species has been studied in detail (Berg and Knutson, 1978), with larval behaviour ranging from quick-killing predators to very specific parasitoids. The species of sciomyzid mentioned here are far from the complete number of sciomyzid species associated with terrestrial snails in southern France and the Iberian Peninsula. These Diptera, however, were the only ones found in the snail species under study. Of the sciomyzids discovered, *Salpicella fasciata* (Fig. 2.1) and *Pherbellia cinerella* were of most initial interest as biological control agents. *S. fasciata* was the first species chosen for an exhaustive study of its life history and snail-killing abilities. Its main host is *T. pisana*, where it lays its eggs quite specifically within the umbilicus of the snail. This specificity was exactly what we were looking for in our control agent and therefore we studied this fly quite extensively. Indeed, Knutson *et al.* (1970) had earlier mentioned the possible value of *S. fasciata* as a biological control agent. However, as we studied its biology further our excitement began to dim as we began to realize that it was not the effective parasite that we had hoped it might be. We discovered that the fly was not very effective at killing the snails that it attacked. When we looked at the size distribution of the snails that the flies were laying their eggs on, we noticed a



Fig. 2.1. Larva of *Salticella fasciata* on *Theba pisana*.

distinct pattern (Coupland and Baker, 1994). The flies were choosing to lay their eggs on large breeding snails, which would most likely die soon. This suggested that the flies were not true (killing) parasitoids but were taking advantage of hosts which were about to provide a source of food for saprophages. The flies could effectively exploit such a food resource by being resident in or on the dying snail. This is a good strategy for the sciomyzid to adopt when competing against some faster-flying sarcophagids, which also utilize the resource of decaying snails. The strategy was, however, obviously not appropriate for a classical biological control agent.

The second sciomyzid which we looked at with great interest was *P. cinerella*, which was very abundant in pastureland, where many of the target snails were found. When we reared *P. cinerella*, we found it to be an active and very efficient predator of the smaller snail species that we were interested in targeting. *P. cinerella* lays its eggs on the soil surface and vegetation in the vicinity of snails. The larvae of *P. cinerella* then search for snails, which they attack and subsequently kill. Unfortunately, we soon realized that *P. cinerella* attacks a large range of snail species. Such a polyphagous predator is not what we wanted to introduce into Australia, where it could potentially attack native endemic snails.

Of the other Diptera associated with the snails, the Sarcophagidae had the greatest diversity of species. At least three species were discovered to be true parasitoids. *Sarcophaga penicillata* (Fig. 2.2) attacked *C. acuta* (Coupland and Baker, 1994) and *Sarcophaga balanina* and *Sarcophaga uncicurva* (Fig. 2.3) attacked both *C. virgata* and *T. pisana*. All three species larviposit within the shell aperture of the snails. A field-captured, adult female *S. uncicurva* larviposited on all available *T. pisana* (25 individuals) when kept in a plastic cage for 1 h. Many of the parasitized snails dropped to the floor of the cage, frothing in an attempt to rid themselves of the larvae.



Fig. 2.2. An adult *Sarcophaga penicillata* attacking a mass of *Cochlicella acuta* snails in Spain.

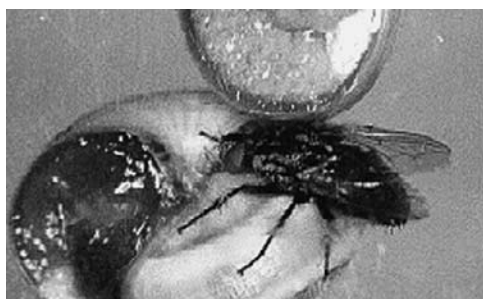


Fig. 2.3. An adult *Sarcophaga uncicurva* attacking *Theba pisana*.

Both *S. uncicurva* and *S. balanina* were imported under quarantine into Australia for host-specificity testing against native Australian snails. However, both parasitoids, whilst highly voracious against *C. virgata* and *T. pisana*, were not considered host-specific enough to risk release there.

A Candidate is Found, Mass Reared, Released and Becomes Established

The most interesting of the sarcophagid species found in Europe was *S. penicillata*, which attacked the conical snails (*Cochlicella* spp.) exclusively. *S. penicillata* larviposits in aestivating snails. The larvae burrow into the snails' flesh, feed and eventually kill their hosts. *S. penicillata* pupates within the snail shell, thus presumably gaining some protection from hyperparasitoids and climatic extremes.

Curiously, when the larvae of *S. penicillata* were made to pupate outside the host snail, the pupal case was often curved, as it would be had it pupated within the snail. *S. penicillata* was sent to Australia for testing, which confirmed its host specificity and suitability for release as a biological control agent of conical snails. It has since been successfully mass reared and released in Australia (Leyson *et al.*, 2003; Lawrence *et al.*, 2004), where it has established in low numbers during the last 4 years. We wait to see if *S. penicillata* will become an effective biocontrol agent. It is common for introduced agents to take some time before suddenly increasing markedly in abundance and becoming effective.

Insights Gained; Finding the Right Candidate can be a Daunting Task

This biological control programme, we believe, can give insights into the problems which may beset other nascent biological control programmes. Indeed, when we began this programme there was very little literature on the biology of potential agents of the snails. At the beginning we were flying blind and had to look at the potential of every agent that we discovered. This was a daunting task as the large numbers of species found to be associated with our targets had to be whittled down to a promising few. This entailed very large-scale rearings of a very diverse range of Diptera. While we were able to quickly shelve many of the agents as unsuitable, it took extensive field studies and cage rearings to dismiss several which, on the face of it, looked very promising. We heavily utilized the taxonomic expertise of the British Natural History Museum and other agencies to identify what we found, and this allowed us to discover more about the known biology of the material we were dealing with. This was immensely helpful in paring down the potential agents to a more reasonable number of species. In the future, it should be apparent to researchers that the search and discovery of suitable biological control agents is a very time- and labour-consuming affair. Funding bodies are often not aware of the extent of the expertise and the physical limitations of early biological control programmes and this should be rectified. Indeed, biological control programmes are really a gestalt between taxonomists, field ecologists and insect-rearing specialists, where the outcome is greater than the individual contributions.

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3

Introductions of Parasitoids to Control the Apple Ermine Moth in British Columbia

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Overview: Two common European parasitoids of the apple ermine moth were released in a classical biological control programme in Canada from 1987 to 1997 after the defoliating invasive species became established in British Columbia in the early 1980s. Species were chosen for release after research by CABI in collaboration with Agriculture and Agri-Food Canada. A parasitoid, *Ageniaspis fuscicollis*, was successfully established in Canada, with parasitism levels as high as 23% recorded in infested areas. Other potential parasitoids considered for release in both Canada and Washington State are discussed.

Apple Ermine Moth Established in Canada

The establishment of the apple ermine moth, *Yponomeuta malinellus* (Lepidoptera: Yponomeutidae) (Fig. 3.1), in North America was probably inevitable; it was found throughout most of the temperate Palaearctic and two infestations had already been recorded, and fortuitously eradicated, in Canada before 1958 (Hewitt, 1917; Parker and Schmidt, 1985). In 1981, this univoltine defoliator of apples was found on nursery stock in Duncan, British Columbia. By 1986 it was known to be spread through southern Vancouver Island, throughout the Fraser Valley on the mainland of British Columbia as well as south of the Canadian/USA border in the Bellingham area of Washington State (Frazer, 1989). By 1994 it had spread further to the commercial apple-producing region in the Okanagan and Similkameen valleys of British Columbia and was known to have established throughout most of Washington and Oregon states to the south (Cossentine and Kuhlmann, 2000).

The apple ermine moth has an interesting life cycle that undoubtedly encourages the ease of its distribution, which is primarily attributed to movement of rootstock. The female oviposits masses of 10 to 80 eggs on the bark of susceptible *Malus* species. First instars overwinter under the egg mass or hibernaculum. Early instars emerge to feed on the upper epidermis and parenchyma of new foliage in the spring (Fig. 3.2), and by the second instar they feed within a communal web. Fourth and fifth instars devour entire leaves, and feeding continues



Fig. 3.1. Apple ermine moth.



Fig. 3.2. Apple ermine moth larvae feeding on apple leaves.

until mid-June, when pupation occurs. The web communities pupate in groups within the now very visible communal web (Menken *et al.*, 1992) (Fig. 3.3). These packs of pupae are easily located to destroy, and the larvae are susceptible to most insecticides registered for use on apple trees, including *Bacillus thuringiensis*. Apple ermine moth infestations on untreated backyard, ornamental or neglected trees are unsightly and because they are rarely treated, contribute to the spread of the insect. The damage is cosmetically, and potentially physiologically and economically, damaging whether the tree is ornamental or fruit bearing. Some infestations have caused complete defoliation of the trees (Antonelli, 1991).



Fig. 3.3. Apple ermine moth pupating within communal web.

The Hunt for Biocontrol Agents

In 1986 the International Institute of Biological Control (now CABI) prepared a literature review of European biological control agents of Yponomeutidae for Agriculture and Agri-Food Canada, which included 60 parasitoids, over 15 predators and some entomopathogens and entomopathogenic nematodes (Affolter and Carl, 1986). Total mean parasitism of *Y. malinellus* found in European studies was estimated to be 30–45%, suggesting that one or more introductions of apple ermine moth parasitoids may be a logical and appropriate strategy to suppress the new pest in Canada. The most common parasitoid in Europe was *Ageniaspis fuscicollis* (Hymenoptera: Encyrtidae) (Fig. 3.4), which had characteristics considered suitable for foreign introduction as it was oligophagous, specific to Yponomeutidae, synchronized with its host species and occupied a wide geographic range (Affolter and Carl, 1986).

Releases of *A. fuscicollis*

Safety measures to anticipate the impact of potential introduced exotic biological controls on ecosystems and non-targets were not yet logical pre-release requirements within the scientific world of biological control of arthropods in the mid-1980s and



Fig. 3.4. An adult *Ageniaspis fuscicollis*.

consequently releases of European *A. fuscicollis* began in Canada in 1987. The scientists carrying out the project, in cooperation with CABI, had the foresight to conduct a study of indigenous apple ermine moth mortality in British Columbia, albeit at the same time as the exotic parasitoid releases (Smith, 1990). The approach to exotic apple ermine moth parasitoid introductions in Washington State was similar, with releases of four parasitoid species from 1988 to 1994 and indigenous biological control agents being recorded in a parallel study (Unruh *et al.*, 1993, 2003).

In the study of British Columbian apple ermine moth populations, it was found that indigenous parasitism of the new pest was minimal, although predation in some locations was high. Egg mass mortality resulted primarily from predation, mostly by a predatory mite, *Balaustium* sp. (Arthropoda: Erthraeidae) (0–35%), although some mortality was attributed to entomophagous fungi (2–4%) and winter slough-off (17–34% of eggs remaining at the end of the summer) (Smith, 1990). Larvae were preyed upon by birds, spiders, ants and earwigs, and 0 to 2.8% of the pupae were killed by four parasitoid species: *Scambus dicorus* and *Ictoplectis quadrangulata* (Hymenoptera: Ichneumonidae), and *Hemisturmia tortricis* and *Compsilura concinnata* (Diptera: Tachinidae). The three latter parasitoids were also recorded in Washington State as being responsible for 2–3% of the pupal mortality (Unruh *et al.*, 1993).

From 1987 to 1990, over 15,700 *A. fuscicollis* were collected in Switzerland, and with 3265 locally reared individuals, these were released in the Fraser Valley and Gulf Islands of British Columbia (Smith, 1990). All imported parasitoids in this study were held under quarantine and inspected for hyperparasitoids before they were released in Canada. Records of insect liberations in Canada are maintained by Agriculture and Agri-Food Canada (e.g. Sarazin, 1988; Sarazin and O'Hara, 1999). Releases resulted in successful establishment of *A. fuscicollis* (Frazer, 1989), although parasitism recorded in the years of these early releases was low: 0–6% parasitism.

A. fuscicollis adults are very small with limited potential for dispersal. It is a univoltine egg parasitoid, although the *A. fuscicollis* eggs do not hatch until the host has reached the third instar (Kuhlmann *et al.*, 1998a). This species' capacity to rapidly increase in numbers would be high as it is also polyembryonic, producing over 80 larvae from a single egg (Junnikkala, 1960). Parasitized hosts are killed in the fifth instar by mummification as the parasitoid larvae pupate. The adults emerge synchronously from the mummified host and immediately mate and begin ovipositing 61 to 224 eggs within the single week that they survive (Kuhlmann *et al.*, 1998a).

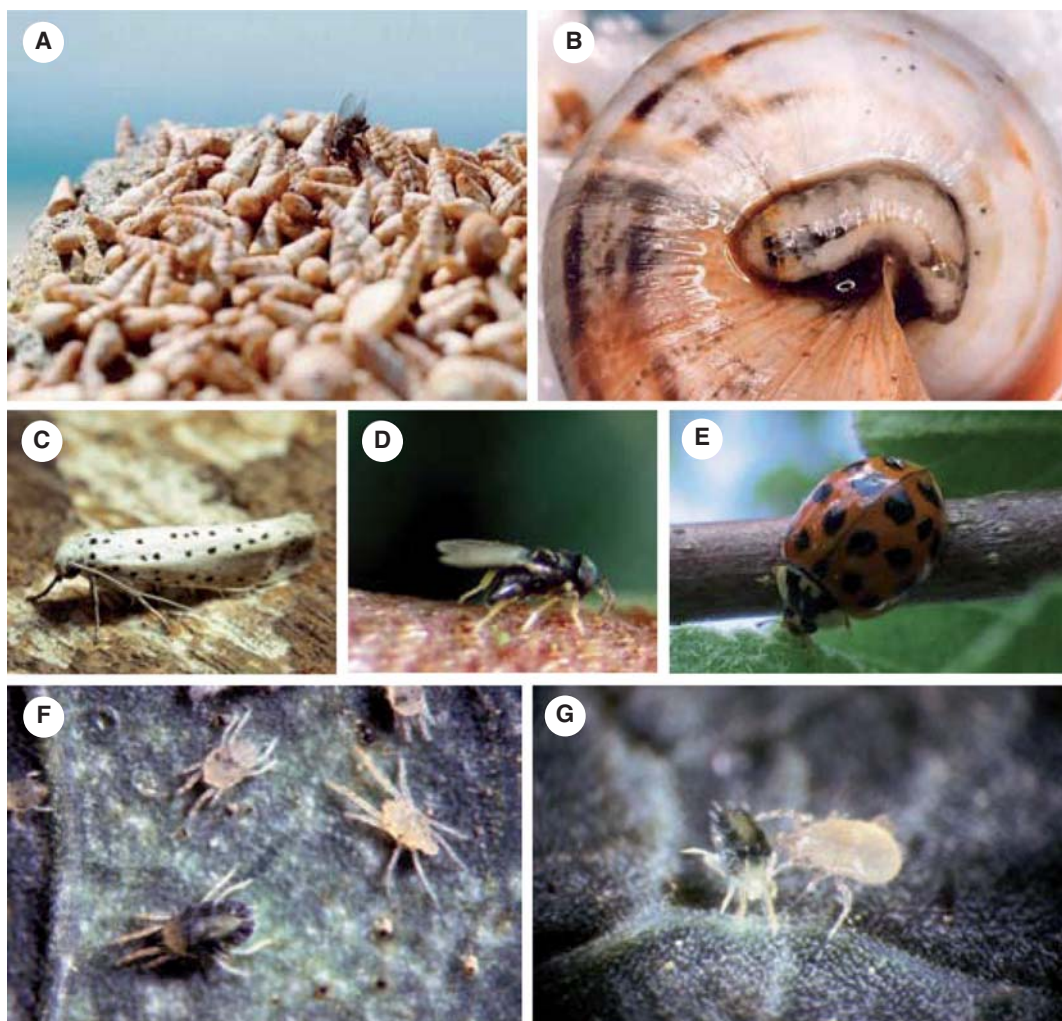


Plate 1.

Figs A and B. Molluscs are the worst agricultural invertebrate pest after insects with slugs attacking grain and horticultural crops across the world. Four species of introduced Mediterranean snails have become serious pests in Australia. Parasites which could be used as classical biological control agents for these invasive pests are being sought (**Chapter 2**). **A.** An adult *Sarcophaga penicillata* attacking a mass of *Cochlicella acuta* snails in Spain. **B.** Larva of *Salticella fasciata* attacking *Theba pisana*. **Figs C and D.** Two European parasitoids of the apple ermine moth were released in a classical biological control programme in Canada after the invasive species became established in British Columbia (**Chapter 3**). **C.** An apple ermine moth. **D.** A parasitoid was successfully established in Canada with parasitism levels as high as 23% recorded in infested areas. **E.** The multicoloured Asian ladybird beetle is one of the most voracious and polyphagous coccinellid predators in the world. It has been introduced in North America as a biocontrol agent to help agriculture. This introduced ladybird beetle has produced some unexpected results (photo Olivier Aubry) (**Chapter 6**). **Figs F and G.** Prior to introduction of biological control agents, the cassava green mite had tremendous negative impact on numerous crops of Africa, notably a 30% reduction in cassava production (**Chapter 5**). **F.** Cassava green mite adult female (lower left), adult male (right), and nymphs feeding on a cassava leaf. **G.** The phytoseiid predator feeding on a cassava green mite adult female.

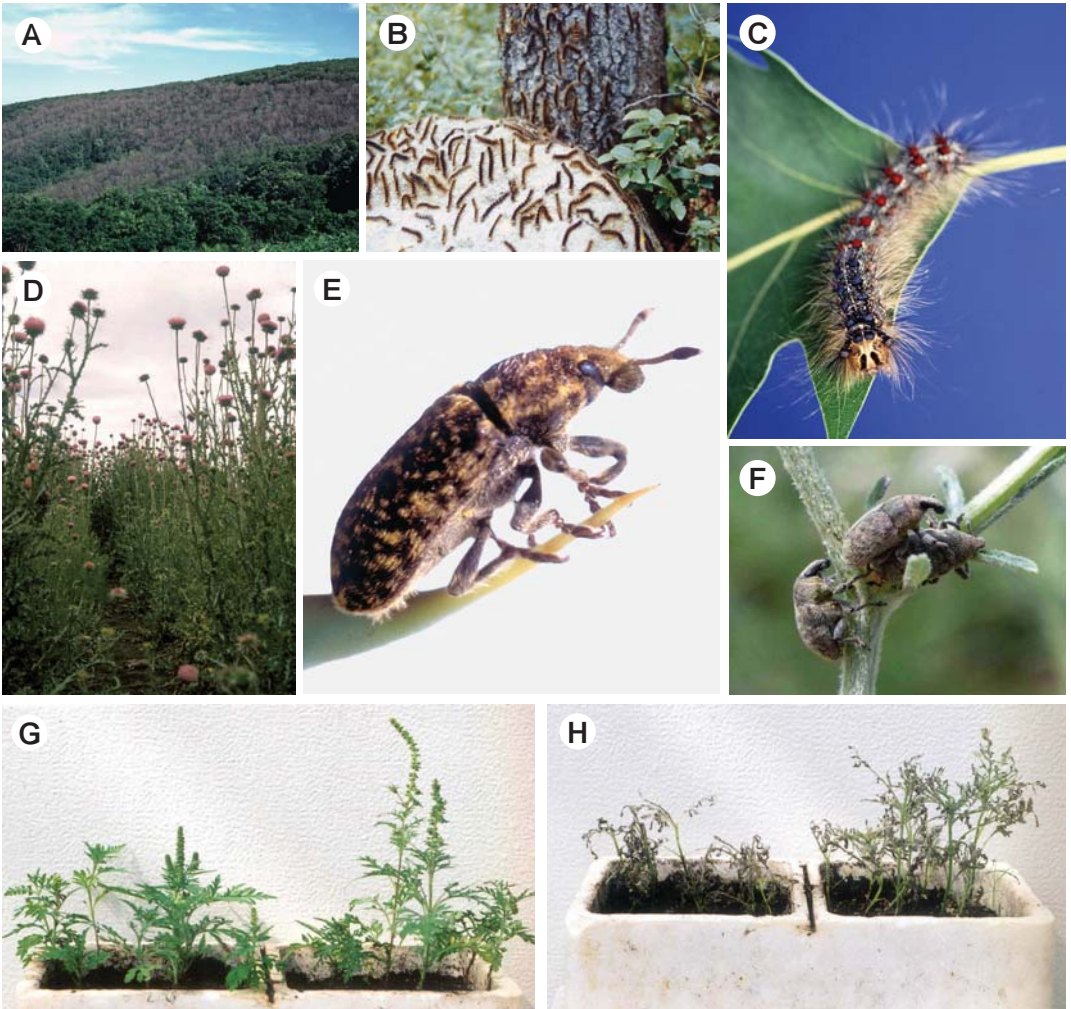


Plate 2.

Figs A to C. Soon after its accidental introduction into North America, the gypsy moth started its spread as an alien invasive species, causing severe defoliation of deciduous forests and shade trees. An entomopathogenic fungus miraculously appeared, decimating gypsy moth populations (**Chapter 7**). **A.** Severe defoliation caused by the caterpillars. **B.** Cadavers of late instar gypsy moth larvae killed by *Entomophaga maimaiga*. **C.** A gypsy moth caterpillar (Photo by Tana Ebaugh) **Figs D and E.** Three species of the Eurasian biennial thistles have established in Canada as noxious invasive species. These thistles are being controlled through introduction of their natural enemies from their original homeland (**Chapter 8**). **D.** High density of nodding thistle in a Canadian pasture prior to the introduction of biological control agents. **E.** *Rhinocyllus conicus*, an agent that successfully controlled nodding thistle following its introduction to Canada. **F.** Following its introduction to North America, diffuse knapweed came to occupy millions of hectares of rangeland. *Larinus minutes* is one species of biological control agent that was introduced for the control of this noxious weed. But is introduction of only one agent enough? (**Chapter 9**). **Figs G and H.** There has been a long and as yet unsuccessful struggle to find suitable biocontrol agents for ragweed, a plant that became a widespread allergenic weed in Eastern Europe (**Chapter 10**). Potted ragweed plants used to test the efficacy of a leaf-eating North American beetle against this noxious weed. **G.** Healthy plants. **H.** The same plants defoliated by *O. communis* larvae in 2 weeks.



Plate 3.

Figs A to H. Purple loosestrife is a European perennial plant that was introduced into the New World almost two centuries ago and is considered a serious weed of wetlands. Public involvement in the rearing and re-distribution of the introduced biological control agents helped in successful classical biological control of this invasive weed (**Chapter 11**). **A.** Adult and **B.** larval *Galerucella californiensis* on a purple loosestrife. **C and D.** Homeowners rearing *Galerucella* in their backyards. (Photo by Jack and Bev Mompson). **E and F.** High School students taking their classroom reared *Galerucella* beetles to a wetland and releasing them. **G.** One of the many Illinois cooperators releasing *Galerucella* beetles into a loosestrife infested wetland. **H.** High school student inoculating her loosestrife plants with *Galerucella* beetles.

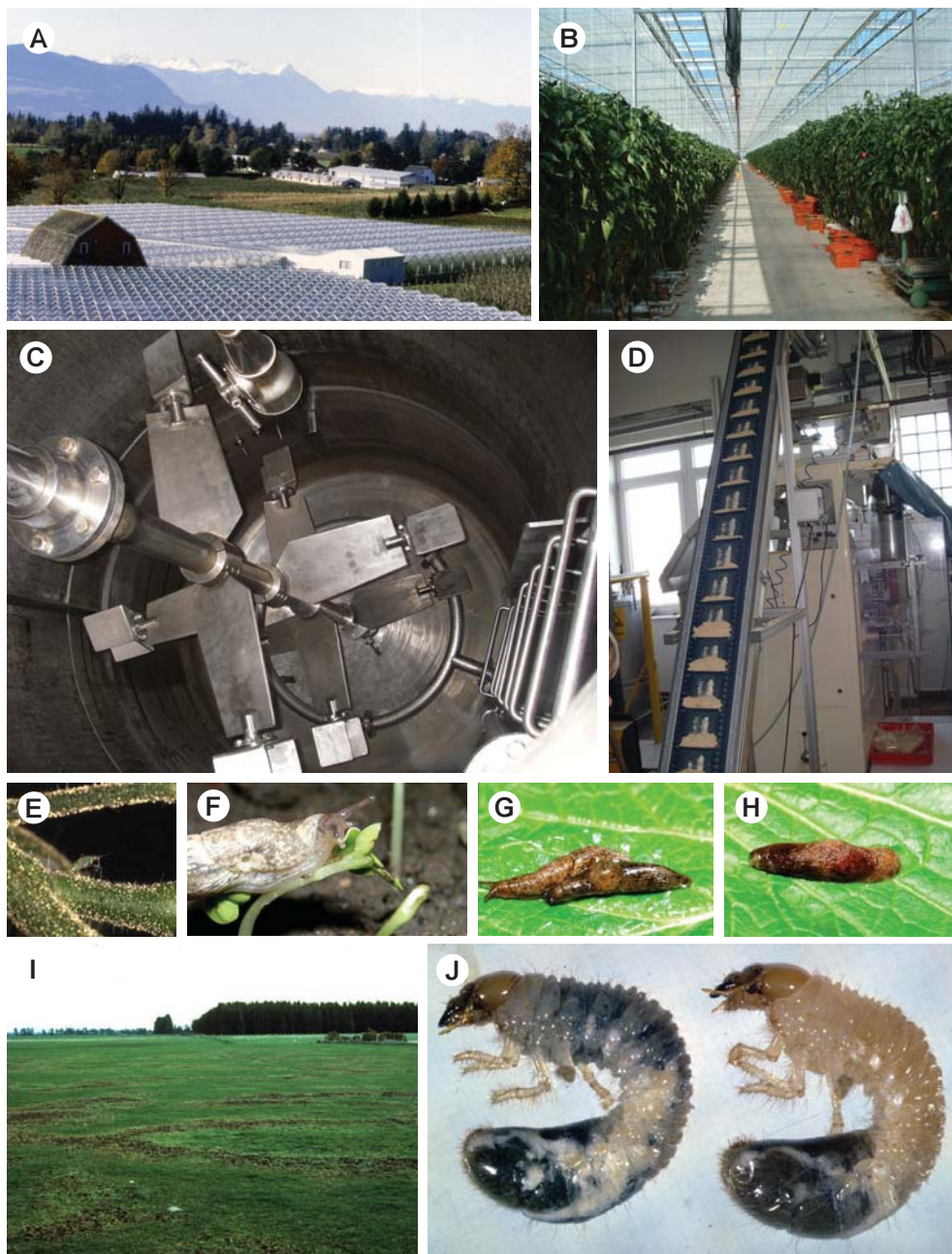


Plate 4.

Figs A and B. Greenhouses provide the ideal setting for biological control (**Chapters 12 and 13**). **A.** A greenhouse operation in Canada. **B.** A pepper greenhouse. **Figs C and D.** Entomopathogenic nematodes have been commercialized as biological control agents of a vast array of pests (**Chapter 15**). **C.** Impeller system in a bioreactor used to mass culture nematodes. **D.** Packing machine used to pack clay-formulated nematodes.

Fig. E. A generalist predator, *Dicyphus hesperus*, is discovered for biological control in greenhouse tomato crops (**Chapter 14**). **Figs F to H.** A novel species of nematode with specific activity against slugs has been developed (**Chapter 16**). **F.** A grey field slug feeding on an oilseed rape (canola) seedling. **G.** Healthy (left) and nematode infected (right) individuals of *Deroceras reticulatum*. **H.** A grey slug killed by the nematode showing nematodes spreading over and feeding on the entire cadaver. **Figs I and J.** A novel bacterium has been commercialized as a microbial pesticide for control of the New Zealand grass grub. (**Chapter 17**). **I.** Damage caused by the grub in New Zealand pastures. **J.** Healthy grass grub larva (left) and amber diseased larvae (right).

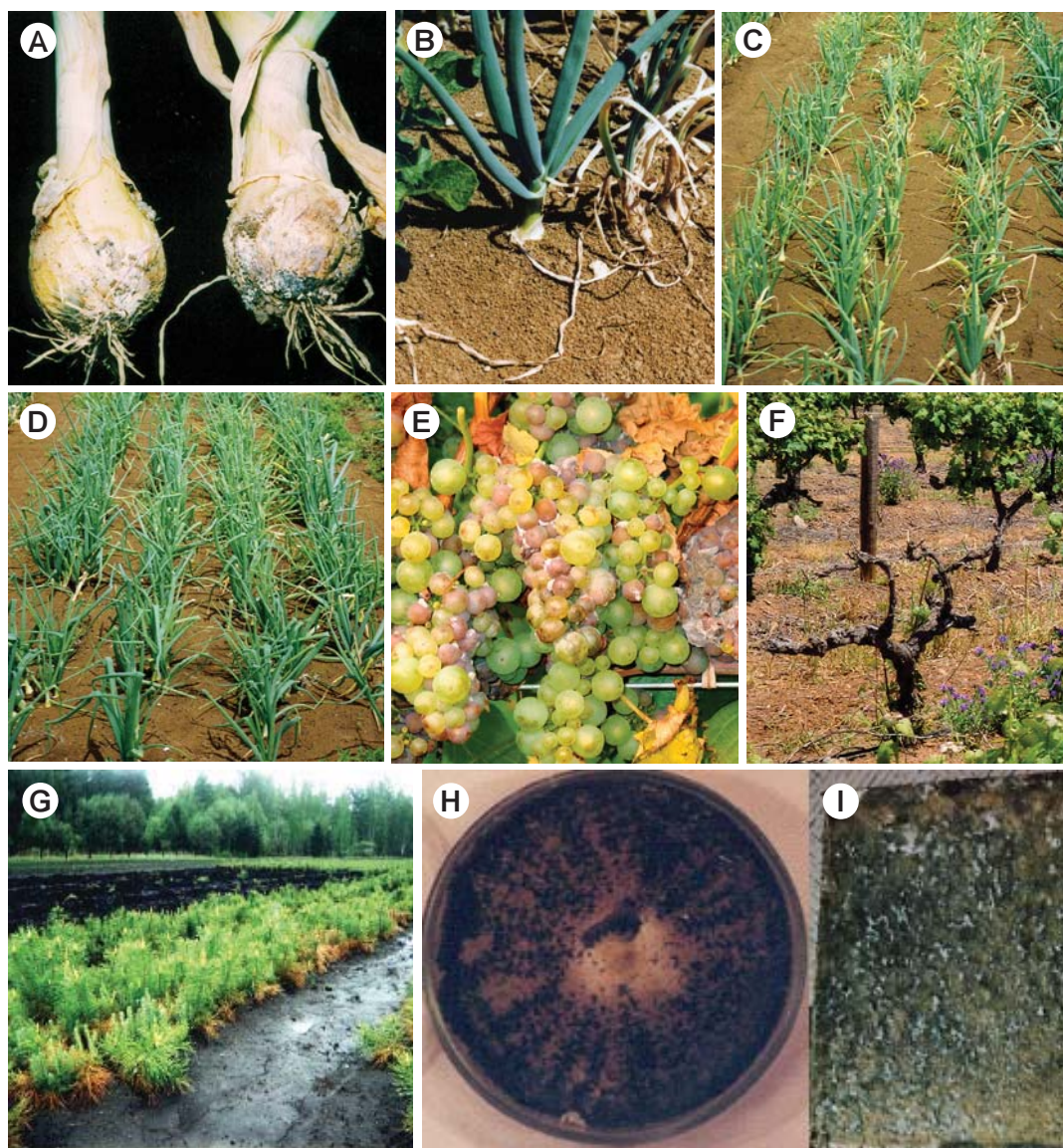


Plate 5.

Figs A to F. The onion industry in New Zealand incurs severe losses from white rot disease caused by the soil-borne pathogen *Sclerotium cepivorum*. A fungal agent *Trichoderma atroviride* LU132 was shown to provide good control not only of this disease, but also of diseases of grapes and other horticultural crops (**Chapter 20**). Onion white rot, *Sclerotium cepivorum* **A.** infecting onion bulbs **B.** disease symptoms in the field. Field trials showing white rot disease control given by *Trichoderma atroviride* pellets, **C.** untreated plot, **D.** treated plot. **E.** Botrytis grey mould on grapes. **F.** Grapevine showing *Eutypa* die-back symptoms. **Figs G to I.** Chemical control of soilborne plant diseases is not permitted in Russia. This has influenced the acceptance of biological control in forest seedling production systems used in reforestation in Siberia (**Chapter 21**). **G.** Damping-off of coniferous seedlings in the field. *Trichoderma asperellum*, **H.** sporulating culture and **I.** formulated conidia of the biofungicide product.

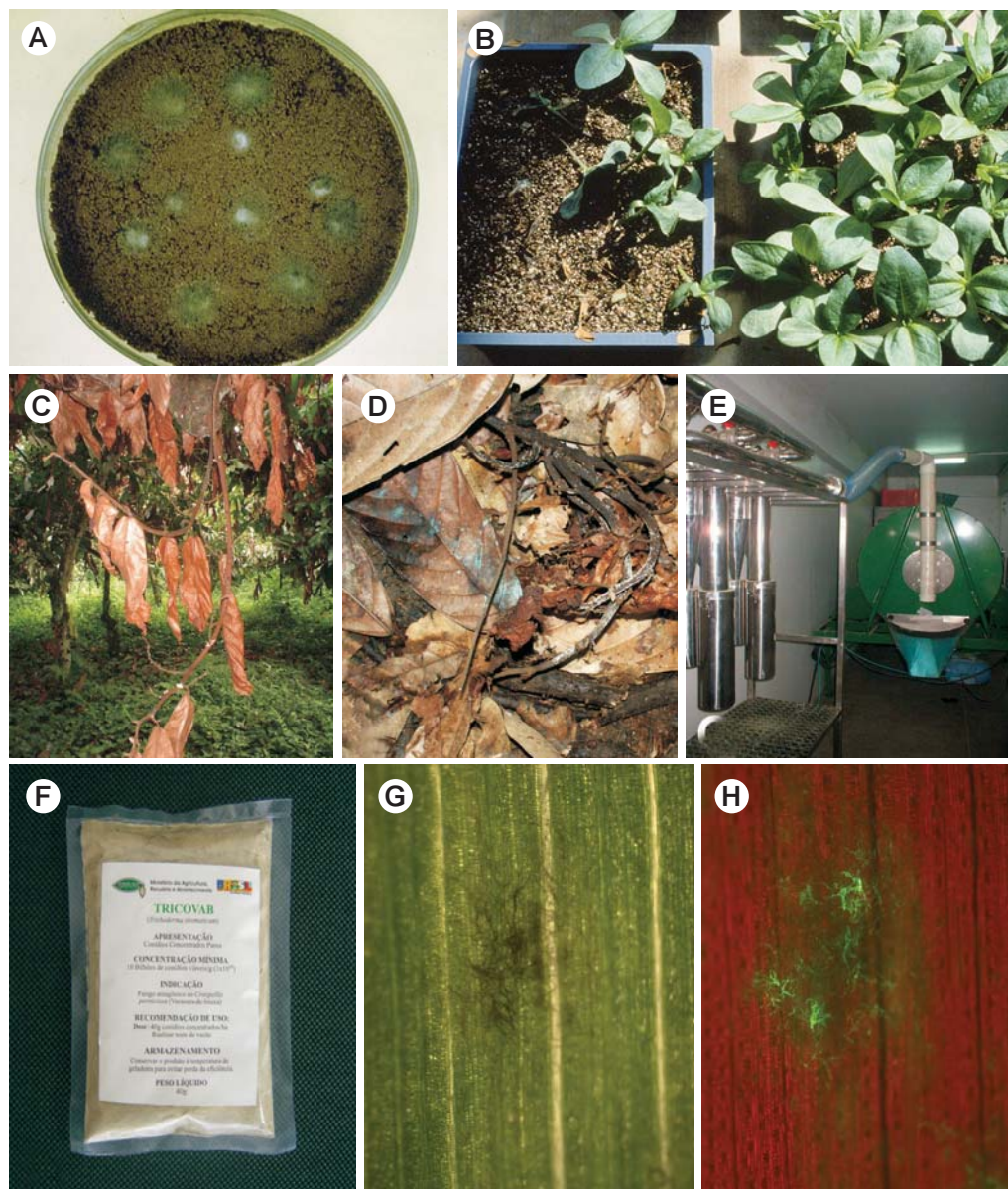


Plate 6

Figs A and B. *Trichoderma* species have been developed as commercial biopesticides to control several soil-borne plant pathogens (**Chapter 22**). **A.** *Trichoderma virens* growing from formulated SoilGard particles on soil. **B.** *Zinnia elegans* seedlings infected with *Rhizoctonia solani* on left, and protected seedlings in soil-less mix amended with SoilGard on right. **Figs C to F.** Witches' broom disease is the main constraint for cacao cultivation in Brazil. *Trichoderma stromaticum* has been developed for control of this weed (**Chapter 23**). **C.** A hanging broom on a cacao tree. **D.** Apparently unimpaired sporulation of *T. stromaticum* on a witches' broom in a plot treated with a copper fungicide (blue-green colour on leaf litter). **E.** Mycoharvester™ used to separate the spores of *T. stromaticum*. **F.** The commercial product, Tricovab. **Figs G and H.** Powdery mildews can cause severe losses to crops under both field and greenhouse conditions. A yeast-like fungus was found to be an exceptional control agent of powdery mildew on a number of crop plants and has been commercialized as the biopesticide Sporodex. Microscope observations of the interaction between a green fluorescent protein-transformed *Pseudozyma flocculosa* strain and the powdery mildew pathogen *Blumeria graminis* f.sp. *tritici* on wheat leaves revealed that *P. flocculosa* is nearly exclusively located in areas where powdery mildew colonies were present (**Chapter 25**).

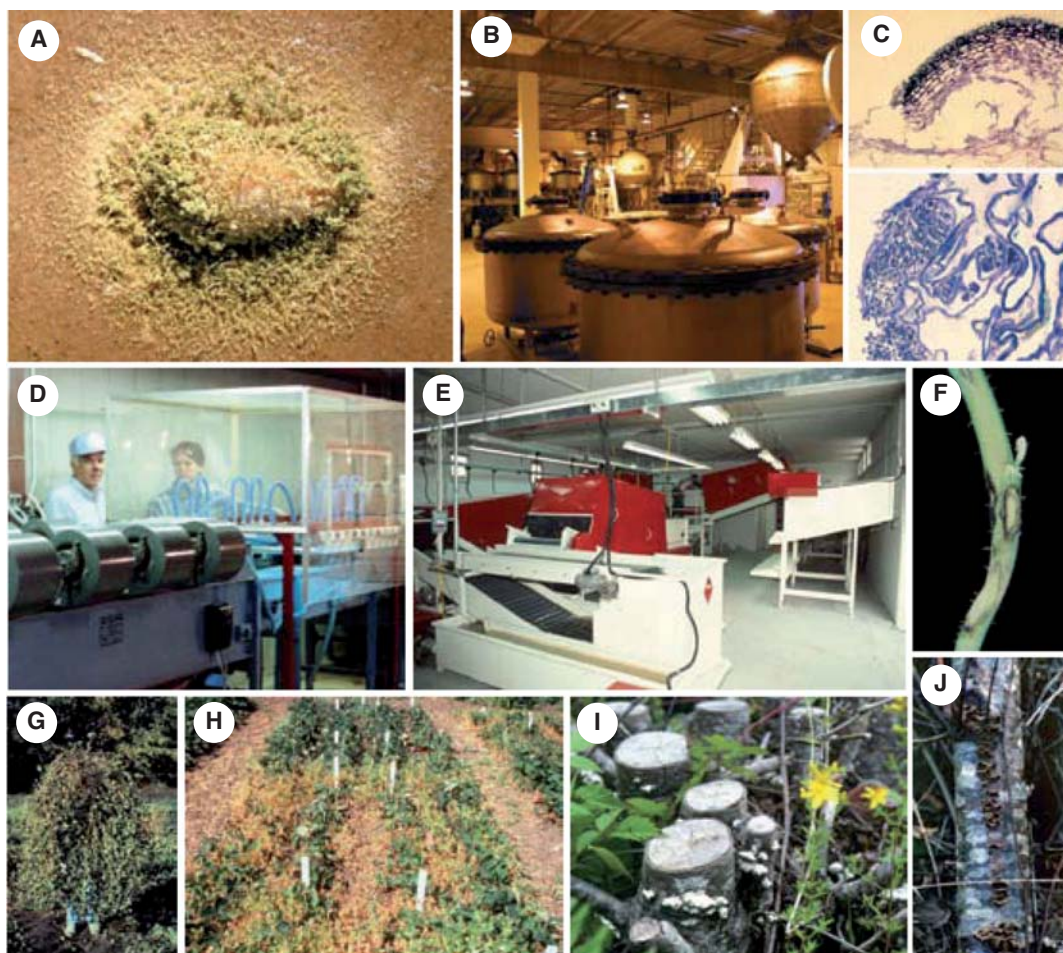


Plate 7

Figs A and B. Aflatoxins are highly toxic, cancer-causing chemicals. *Aspergillus flavus* is the most important causal agent of crop aflatoxin contamination. A strategy for preventing aflatoxin contamination based on the use of naturally occurring isolates of *A. flavus* that lack aflatoxin-producing ability (atoxic strains) was developed (**Chapter 27**). **A.** Atoxic strain *Aspergillus flavus* AF36 growing out from a colonized wheat seed 7 days after application. **B.** The manufacturing room of the atoxic strain production facility. **Figs C to E.** Biological control of postharvest products has great potential because postharvest environment parameters can be rigidly controlled to suit the needs of the biocontrol agent. New biological control products were discovered and developed to control these postharvest diseases. **C.** *Candida oleophila* forming a film along surface of wound in apple. **D and E.** Semi-commercial lines used to evaluate potential antagonists, formulated products, and combined treatments. **Figs F to H.** *Colletotrichum gloeosporioides* f. sp. *malvae* was discovered by fortuitous observation as blight on seedlings of round-leaved mallow (*Malva pusilla*), a serious weed pest in prairie agriculture. This fungus has now been developed as the bioherbicide, BioMal® (**Chapter 30**). **F.** Disease symptoms on round-leaved mallow stems caused by *Colletotrichum gloeosporioides* f.sp. *malvae*. **G.** Round-leaved mallow showing long branches and prolific seed production. **H.** Control of round-leaved mallow in strawberry plots treated with *Colletotrichum gloeosporioides* f.sp. *malvae*. **Figs I & J.** *Chondrostereum purpureum* effectively prevents sprouting of cut stumps of deciduous, but not coniferous, trees by colonizing and decaying their stumps. This fungus has been successfully developed as a control agent of woody deciduous weeds where brush control is required (**Chapter 31**). **I.** Cutting stems of alder followed by treatment with *Chondrostereum purpureum* resulted in the complete suppression of re-sprouting from the cluster of stems. The fruiting structures were observed on the dead stems approximately 18 months following application. **J.** Fruiting structures of *Chondrostereum purpureum*, commonly found on wounded stems of deciduous trees. The stems above the site of infection usually die-off while those below the site of infection may continue growing.



Plate 8.

Figs A to H. Pine caterpillars are common forest pests in China causing severe defoliation and economic loss. For the last 36 years, *Beauveria bassiana* has been used for pine caterpillar management in China (**Chapter 33**).

A. Masson's pine caterpillar, *Dendrolimus punctatus*. **B.** A cadaver of the Masson's pine caterpillar infected by *B. bassiana*. **C.** Shallow tray culture of *B. bassiana* in a small plant in the 1970s and 1980s. **D.** Industrial solid fermentors used for production in the early 2000s. **E.** Mist spray application in the 1970s. **F.** Application by explosion in the 1970s. **G.** Firework mortars for conidial powder application in 1975. **H.** A newly developed mortar for launching the fireworks of *B. bassiana*.

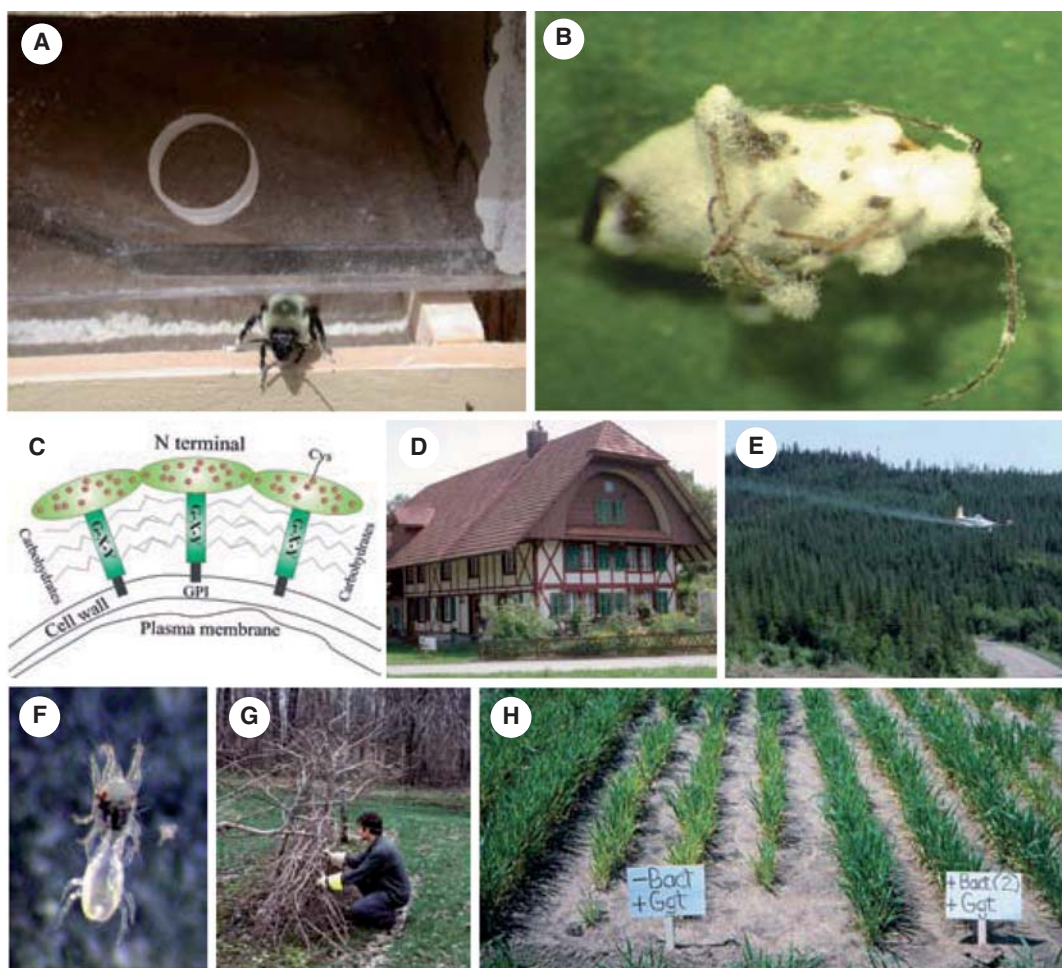


Plate 9.

Figs A and B. Pollinating and flower visiting insects can carry some plant diseases, and can themselves be infected while foraging at flowers. Pollinators are being considered as carriers and disseminators of microbial biocontrol agents (**Chapter 35**). **A.** A bumblebee sitting on the edge of a dispenser coated with inoculum. **B.** A dead Lygus bug killed by a fungal pathogen that was brought to the host by a bee. **Fig C.** Pathogenic fungi use a unique molecule to hide themselves from the host immune system (**Chapter 36**). **Fig D.** Early Andermatt BIOCONTROL laboratories in a traditional Swiss farmhouse. The first preparations of a viral biopesticide were prepared in the bathtub of a student's dorm (**Chapter 37**). **Fig E.** Outbreak populations of balsam fir sawflies have been severe in western Newfoundland. A species-specific nucleopolyhedrovirus has been developed for use in operational control programs directed against this forest insect pest. Here, an FPL Cessna 188 sprays the virus over a Newfoundland forest (Photo by S. Holmes) (**Chapter 39**). **Figs F and G.** (Photos by J. Lasnier). There are over 100 different species of arthropods that are direct or indirect pests of apple. A successful biological control programme has been developed for phytophagous mites, using inoculation and conservation measures of predators (**Chapter 41**). **F.** An adult predatory mite feeding on an adult two-spotted spider mite. **G.** Placement of winter pruned wood at the base of recipient trees allows for conservation and dissemination of the beneficial predatory mites. **Fig. H.** The discovery that take-all, a root disease of wheat and barley caused by the fungus *Gaeumannomyces graminis* var. *tritici* (Ggt) declines in severity and can all but disappear where wheat is grown in the same field over many years provides an exemplary model system for studying soil ecosystems that become 'disease suppressive'. Here, a field test shows the suppressiveness of bacterial strains to take-all when applied as a mixture to the seeds of wheat with the pathogen (Ggt) introduced into the seed furrow (+Bact + Ggt) compared to the pathogen added to the seed furrows with no seed-applied bacteria (-Bact + Ggt) (**Chapter 44**).

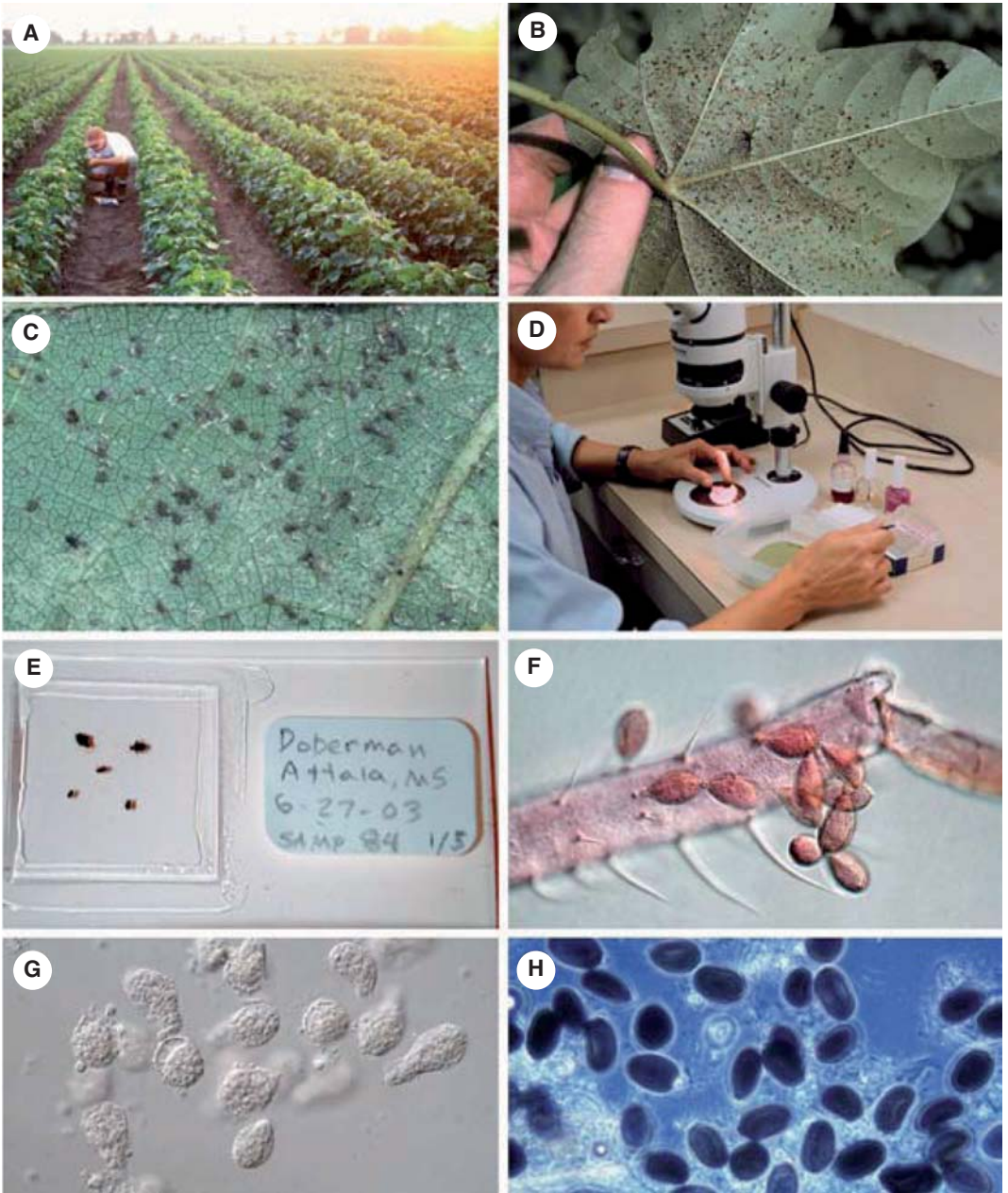


Plate 10

Figs A to H. Avoiding application of insecticides may sometimes allow naturally occurring biological control agents to hold pests in check as is the case with cotton aphid populations, which are often decimated by a fungal epizootic (**Chapter 42**). **A.** Collecting aphid-infested cotton leaves. **B.** Extremely high population of cotton aphids. **C.** Fungal epizootics frequently kill nearly all cotton aphids in a cotton field within a few days. All that remains are dead aphids covered with saprophytic fungi. **D.** Random subsamples of cotton aphids from the field are brought to the laboratory for fungal diagnosis. **E.** Cotton aphids are small, soft, easily mounted and squashed on slides making precise diagnosis possible for each aphid. **F.** Leg of a cotton aphid showing the distinctive and firmly attached spores of the pathogenic fungus. **G.** When infected aphids are squashed, protoplasts or hyphal bodies of the fungus are released from the haemocoel. **H.** Resting spores of the fungus are liberated from the haemocoel of an infected cotton aphid and serve to infect future generations of the aphid.

In 1995 a study of the significance of natural enemies in European apple ermine moth populations was carried out by CABI in collaboration with Agriculture and Agri-Food Canada. Data from this work indicated that *A. fuscicollis* parasitism was independent of host density at the whole tree level, but at the individual web scale, the probability of a communal web containing parasitized host larvae increased and the percent parasitism decreased with the number of host larvae per web (Kuhlmann *et al.*, 1998b). From 1995 to 1998, 104,923 additional *A. fuscicollis* were collected from Europe and released on Vancouver and Salt Spring Islands, in the Fraser Valley and in the Okanagan and Similkameen valleys of British Columbia (Cossentine and Kuhlmann, 1999). Mean parasitism by *A. fuscicollis* increased and was as high as 23% on Vancouver Island, where apple ermine moth was consistently found. Apple ermine moth populations have decreased in areas where *A. fuscicollis* was released, although it is not certain that the parasitoid is entirely responsible for the reduction in population densities (Cossentine and Kuhlmann, 2001).

Releases of *H. brunnicornis*

The second apple ermine moth parasitoid chosen for release in Canada was *Herpestomus brunnicornis* (Hymenoptera: Ichneumonidae). This is a solitary, univoltine endoparasitoid of fourth and fifth instars and pupae (Kuhlmann, 1996). This parasitoid species is specific to hosts in the genus *Yponomeuta*, it is well synchronized with its host and it occupies a wide geographic range (Affolter and Carl, 1986). In an investigative study by Kuhlmann (1996), overwintered adult female *H. brunnicornis* were found to parasitize apple ermine moth in an inverse density-dependent manner. The females are synovigenic and a limited number of eggs mature each day. Female handling time of host pupae is also high. Overwintering females must host feed before they are able to successfully parasitize a host, and a large number of hosts are killed in this way. In 1990, 438 *H. brunnicornis* emerging from apple ermine moth collected in Japan and Europe were released in the Fraser River Valley and on Vancouver and Galiano Islands and 4010 female wasps were released on Vancouver Island in 1998. In 1996 and 1997, 3225 *H. brunnicornis* females collected in Switzerland were released in apple ermine moth-infested orchards in the Okanagan and Similkameen valleys of British Columbia. This parasitoid has not yet been confirmed to have successfully established in the apple ermine moth populations in British Columbia. The establishment of *H. brunnicornis* in Washington State where it was released is also questionable, although it was reported as being recovered at low rates in 1994–1995 (Unruh *et al.*, 2003).

Biocontrol Agents Considered Unsuitable

Diadegma armillata (Hymenoptera: Ichneumonidae) was also considered as a potential species to introduce in British Columbia; however, as a polyphagous parasitoid of microlepidoptera it was deemed unsuitable for introduction (Herting

and Simmonds, 1982). *D. armillata* was released in Washington State and is recorded as recovered at one release site to date (Unruh *et al.*, 2003). *Eurystheae scutellaris* (Diptera: Tachinidae) was also released in Washington State (Unruh *et al.*, 2003). The predatory fly, *Agria mamillata*, was considered for release in Canada (Kuhlmann, 1995) but no releases were made.

Lessons Learned

In describing the classical introduction of two European-derived parasitoids of the apple ermine moth into Canada for the purpose of biologically controlling the new exotic apple ermine moth, it is apparent that although it was a relatively well-executed introduction, it was not based on the safety precautions currently in place to protect against the release of destructive new species. The fact that similar studies of the pest were carried out in the USA and that the same two parasitoid species were chosen for release on both sides of the Canadian/USA border demonstrates that international introductions could probably be better handled through collaborative classical biological control projects, particularly as parasitoids do not recognize human-imposed borders. Finally, it is important that we find the time and funding within ongoing biological control studies to follow the status of imported biological controls that have been carefully released into our ecosystems.

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4

Introductions of Parasitoids to Control the Imported Cabbageworm

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Overview: Three main parasitoids have been released against imported cabbageworm. Only *Cotesia rubecula* has been demonstrated experimentally to reduce pest populations, although life-table studies suggest that *Cotesia glomerata* is an important source of mortality in the USA. Population range of one native non-target butterfly was reduced in the northeastern USA. Weakness in evaluation occurred because introductions of natural enemies were often goals by themselves, rather than part of a bigger project incorporating pre- and post-introduction evaluations on the target and related non-target butterflies.

The Cabbage Butterfly

Pieris rapae (Lepidoptera: Pieridae) is a European butterfly that infests garden cabbage and other cole crops. It invaded temperate regions around the world, reaching Canada by 1860 (Scudder, 1889) and rapidly becoming one of the most common urban butterflies in temperate North America. Because of the highly visible impact of larval feeding on cabbage, control has been necessary in commercial crops and home gardens, causing the butterfly's management to be extensively investigated. While large populations are usually associated with crops, smaller numbers survive over much larger areas on native and introduced crucifers in disturbed areas. Unlike the native species of *Pieris* butterflies in the northeastern USA, *P. rapae* does not fly in shaded forest habitats, but remains in sunny habitats like meadows, roadsides and non-forested river edges, where the annual or biennial crucifer species it feeds on occur.

Attempts at Biological Control

Because of the damage caused by *P. rapae* larvae, efforts to reduce its density with classical biological control began soon after the pest's invasion of the USA and were later repeated in several other countries. These efforts, some as old as the 1880s and others that I undertook in the 1980s, provide several important

lessons for how classical biological control should be done to meet modern standards. In this chapter I focus on three main questions: (i) did the project work? Is there evidence that the pest's density was reduced; (ii) did parasitoids introduced against *P. rapae* harm native butterfly populations; and (iii) if the project were being done fresh today, could the choices of which species of parasitoid to introduce and what source locations to collect them from be improved? Answers to these questions can help us do a better job in future biological control projects. My involvement in the project spans a period of research from 1985 to 2005 and includes both being a participant in the biological control project against *P. rapae* (I introduced the Chinese population of the solitary larval parasitoid, as discussed below) and investigating potential field impacts of the introduced parasitoids on native butterflies.

Four parasitoids were released in North America against *P. rapae*. Two of these are braconids that attack young larvae: *Cotesia glomerata* and *Cotesia rubecula*. One is a pteromalid that parasitizes pupae: *Pteromalus puparum*, and one is an egg parasitoid: *Trichogramma evanescens* (Clausen, 1978; Van Driesche and Nunn, 2002). The first three have become widely established. Using one or more of these parasitoids, classical biological control of *P. rapae* was attempted in Canada, the USA, New Zealand, Australia, Bermuda and the Philippines. *C. glomerata* was also released in Chile in the 1970s, against the related invasive butterfly *Pieris brassicae* (Ripa and Rojas, 1994). While release of *C. glomerata* in Chile has also had damaging consequences for non-target butterflies, I limit my discussion to efforts directed against *P. rapae*, mainly in the USA and New Zealand.

When the USDA (USA) decided to attempt biological control of *P. rapae* in the 1880s, entomologists selected the gregarious larval parasitoid *C. glomerata* (formerly *Apanteles glomeratus*) as the natural enemy to be imported first. After some unsuccessful attempts, material taken from England was established in 1884 near Washington, DC (Clausen, 1978). In Europe, *C. glomerata* is a parasitoid of *P. brassicae* that occasionally attacks *P. rapae* (Puttler *et al.*, 1970; Laing and Levin, 1982). Consequently, it was not a good choice of species for the intended target. It is, however, gregarious, laying up to 50 eggs per host in first and second instars, the preferred host stage, and the large number of cocoons spun by larvae as they emerge from 5th instar *P. rapae* probably attracted attention to this species as a candidate agent. The status of *C. rubecula* as the more specialized *P. rapae* parasitoid seems to have been missed at this stage of the project. The field hosts of *C. glomerata* include *Pieris napi* in Britain (Lees and Archer, 1974), Japan (Sato and Ohsaki, 1987) and the northeastern USA (Benson *et al.*, 2003a). Other known field hosts are *Pieris protodice*, *Pieris melete* and *Aporia crataegi* (Krombein *et al.*, 1979; Laing and Levin, 1982; Ohsaki and Sato, 1990; Jiang ShuangLin, 2001), and, in Chile, *Tatochila autodice blanchardii* and *Tatochila mercedis mercedis* (A. Sharp, personal communication). Under laboratory conditions, *Pieris virginiensis* is a suitable host (Benson *et al.*, 2003b).

In contrast to *C. glomerata*, *C. rubecula* is a solitary, more specialized parasitoid of *P. rapae* in Eurasia, including China, which kills caterpillars as fourth instars. Interest in this species in North America began when a self-introduced population was discovered in British Columbia in the 1960s. This strain, the first

of three to be introduced into the USA, was released in Missouri, New Jersey, South Carolina and Ontario (Puttler *et al.*, 1970). It failed to establish in Missouri, but may have done so in Ontario (Corrigan, 1982). After the fact, this poor establishment record was explained by an improperly timed diapause-induction response in this British Columbian strain (Nealis, 1985), which probably originated from England or another area with similar climate. To try to solve this problem, an effort was made by the USDA to collect *C. rubecula* in Europe from an area with a continental climate, where latitude and winter temperatures were more similar to those of the cooler parts of the eastern USA. A second strain was obtained from the former Yugoslavia and released in Missouri, Virginia and Ontario. It was recovered in Virginia in 1988 but apparently died out due to hyperparasitism. In 1993, *C. rubecula*, of uncertain origin, was found to be the dominant *P. rapae* parasitoid in Quebec. When I became interested in this project in the mid-1980s, I noted literature citations that recorded *C. rubecula* as a parasitoid of *P. rapae* in northern China, a region that is better matched climatically to eastern North America than is Western Europe (because of the confounding effect in Europe of warming by the Gulf Stream). Consequently, I reasoned that this would be a very good source from which to import the parasitoid for establishment in New England (USA). A population from Beijing, China, was collected and released from 1988 to 1993 in Massachusetts, Connecticut and Rhode Island at 17 locations and easily and rapidly became established and self-spreading (Van Driesche and Nunn, 2002).

The choice of *C. rubecula* for use against *P. rapae* was much better justified than *C. glomerata*, based on its known field host range, which is limited to *P. rapae* (Puttler *et al.*, 1970; Benson *et al.*, 2003a). However, in laboratory tests, *C. rubecula* does oviposit and successfully develop in other butterflies, including *P. virginiensis* (Benson *et al.* 2003b), *P. napi* and *P. brassicae* (Geervliet and Brodeur, 1992). The release of this species preceded host range reviews for parasitoids (which started in the USA in the mid-1990s) and if such a review had been conducted these laboratory findings would have probably been interpreted as an indication of potential non-target impacts. In practice, no field use of these hosts has occurred.

The other two parasitoids released against *P. rapae* in North America – *P. puparum* and *T. evanescens* – had little impact and I discuss them briefly only for sake of completeness. *P. puparum* is actually native to North America, having been recorded in Canada in 1844 (Scudder, 1889). It is a polyphagous pupal parasitoid that attacks Lepidoptera in several families. Because of its polyphagous nature, this species was not a good choice as a biological control agent. However, in North America, its accidental introduction had no practical consequences as it was already present. Unfortunately, it became part of the 'formula' for releases against *P. rapae* and was later deliberately introduced into New Zealand, Australia and Bermuda (Clausen, 1978). Relatively high rates of parasitism (33–64%) of *P. rapae* pupae by *P. puparum* have been recorded in unsprayed cole crops in Virginia, New Zealand and Australia (33–36%), but the population impact of this parasitism has not been measured.

The other parasitoid released against *P. rapae* in North America was *T. evanescens*, which apparently established in both California and Missouri

(Clausen, 1978). This species potentially could be important, since in China it is a common native parasitoid of *P. rapae* eggs (Zheng and Li, 1983). Studies of its effects on *P. rapae* and other non-target species in North America, however, have not been conducted.

Was the Pest Controlled?

The most basic question in a classical biological control programme – was the pest controlled? – can sometimes also be the most difficult to answer. Methods to make such determinations come down to either comparing pest density in plots having and lacking the natural enemy whose impact is to be scored or constructing life tables for the pest and seeing what role the natural enemy seems to play in the life table. The best moment for use of the first approach is when a new natural enemy is first being introduced. For *C. glomerata* in North America, no efforts were made in the 1880s to do such an evaluation. This leaves only sample parasitism values in study plots or the construction of life tables as routes to estimate the impact of *C. glomerata*. In terms of simple sample parasitism values, *C. glomerata* in Massachusetts prior to the introduction of *C. rubecula* in 1988 looked important, parasitizing 60–80% of larvae in unsprayed cole crops (Van Driesche, 1988). Life-table studies confirmed that this parasitoid really was an important mortality factor (Van Driesche and Bellows, 1988), but the level of control provided was usually not sufficient by itself for commercial crop production. No evaluation of the impact of *C. rubecula* on *P. rapae* has yet been done in North America. However, studies carried out in New Zealand at the time of the parasitoid's introduction clearly demonstrated that *C. rubecula* lowered the density of 5th instar *P. rapae* larvae significantly (Cameron and Walker, 2002). Parasitism in plots with *C. rubecula* was consistently above 70% and densities of 5th instar *P. rapae* at harvest were reduced by 85%, from 1.65 per plant in control plots to only 0.25, greatly reducing plant damage. While not enough was done to evaluate the impact of the biological control programme against *P. rapae* in all locations, the above-cited data do indicate significant pest reduction, at least from *C. rubecula* in New Zealand.

An important feature of this biological control project in North America, Australia and New Zealand is that two larval parasitoids were released that were likely to compete strongly with each other. Since all parasitoid introductions involve some potential risk to non-target species, it is of interest to be able to forecast which of any given set of candidate natural enemies might be the superior agent. For the *P. rapae* project, field data clearly show that establishment of *C. rubecula* has caused a decline in the density of *C. glomerata*. This was detected wherever careful studies were conducted, including Oregon and Washington (Biever, 1992), Massachusetts (Van Driesche and Nunn, 2002), and New Zealand (Cameron and Walker, 2002). In New Zealand, in plots without *C. rubecula*, parasitism by *C. glomerata* was 10–60%, but this declined to < 10% when *C. rubecula* was present (Cameron and Walker, 2002). In Massachusetts, densities of *C. glomerata* cocoon masses after release of *C. rubecula* declined by 81%,

with only 3% (82/2706) of the collard plants in an unsprayed patch bearing *C. glomerata* cocoons, compared to 16% (661/4098) of plants with *C. glomerata* in the same plot in 2 years before the release of *C. rubecula* (Van Driesche and Nunn, 2002). Laboratory studies predict this outcome, since *C. rubecula* first instars have mandibles while those of *C. glomerata* do not. This allows *C. rubecula* to kill competing larvae (Laing and Corrigan, 1987), making it the intrinsically superior competitor. That *C. rubecula* can suppress *C. glomerata* is information that could have been used to select *C. rubecula* as the parasitoid of choice for introduction in the first place. Also, this outcome has implications for non-target butterfly conservation because *C. glomerata* appears to have had unwanted effects on some non-target pierids (see next section), while *C. rubecula* does not. The introduction of *C. rubecula* is therefore beneficial not only because it better suppresses the pest but also because it reduces harm to non-target species by *C. glomerata*.

Impacts on Non-target Butterflies

The second important question that I wanted to answer about this biological control project was whether it had caused harm to native butterflies. At least two of the four parasitoid species introduced have been found attacking native butterflies. *P. puparum* in New Zealand attacks the endemic red admiral (*Bassaris gonerilla*), and *C. glomerata* parasitizes *P. napi* subspecies in California (Shapiro, 1981) and New England (USA) (Benson *et al.*, 2003a). In Massachusetts, *C. glomerata* actually prefers to attack the native species *P. napi oleracea* over *P. rapae* by a ratio of nearly five to one (Van Driesche *et al.*, 2003). Levels of parasitism of the second brood of *P. napi* found in meadows are so high (66–100%) (Van Driesche *et al.*, 2004) that few if any larvae are likely to become overwintering pupae.

Lessons Learned

The third question to ask of any biological control project is what can be learned from it to help improve future work. I see five lessons in this case.

1. Studies of a pest's natural enemies in their native range should precede the choice of particular parasitoids for field release.
2. Local populations of a species should be treated as separate entities during consideration for release to new areas.
3. Natural enemies should be collected and released from climatically similar areas.
4. Host ranges of candidate agents should be estimated and polyphagous parasitoids not released.
5. Impact of released agents on the target pest should be measured.

1. This project was flawed by a rush to judgment in the 1880s that *C. glomerata* was a parasitoid suitable for release against *P. rapae*. This parasitoid was introduced to North America because it was found attacking *P. rapae* in Europe and was gregarious (and thus many parasitoids could be quickly collected). That it was not primarily a parasitoid of *P. rapae*, but rather of *P. brassicae*, was overlooked or ignored. Selection of *C. glomerata* diverted attention away from *C. rubecula*, delaying its introduction to North America by more than 80 years. Host/parasitoid studies in the pest's native range (Europe) before committing to particular introductions would have shown that both *C. glomerata* and *P. puparum* were polyphagous and that *C. glomerata* did not usually attack *P. rapae*. Rushing to make a quick introduction before gaining such needed information was poor science.
2. A second lesson from this project is that populations, not species, are what get introduced. Populations within a single species can vary strongly in characters that affect them as biological control agents, including their climatic adaptations and host specificity. For example, in Western Europe, *C. glomerata* is predominantly a parasitoid of *P. brassicae*, attacking *P. rapae* only occasionally. But in Japan, where *P. rapae* and *C. glomerata* occur but *P. brassicae* was absent until its recent invasion, *C. glomerata* is unable to even complete its development in *P. brassicae* (Sato and Ohsaki, 2004). These two *C. glomerata* populations, while given the same species name, are functionally different entities in terms of their potential roles in biological control projects. Consequently, only the exact population whose host specificity is determined through testing in the project should be used as a source from which to collect for release.
3. A third lesson is that the potential for climatic mismatch to cause an introduction to fail is real and must be considered in choosing where to collect the natural enemies used in a project. The Vancouver strain of *C. rubecula* failed to establish in the eastern USA and the Yugoslavian strain required decades before incipient established populations increased and spread. In contrast, the strain from near Beijing, China, released in Massachusetts established immediately and spread rapidly. *C. rubecula* populations vary in how short the day length must be to trigger diapause. At higher latitudes, when parasitoids enter diapause as days shorten, they do so at day lengths that are still relatively long in absolute terms compared to lower latitudes. When such parasitoids are moved to lower latitudes, they misinterpret days of the same length as still meaning the onset of cold weather, even though such day lengths happen earlier in the season when temperatures are still mild. Such mismatch caused some strains of *C. rubecula* to enter diapause prematurely (in California, Missouri and Virginia) before seasonal temperatures were sufficiently low (Nealis, 1985). If parasitoids enter diapause too early, they consume their fat stores rapidly due to the high temperature and later die in winter, not due to cold but from lack of fat reserves. To avoid such problems, sources and release locations should have similar climate and latitude when dealing with natural enemy species likely to use seasonal photoperiods to regulate their diapause or other critical life-history events.
4. Native insects have recently been recognized as wildlife meriting conservation in their own right. If the field hosts of the parasitoids used in this project had been studied in Europe, those data could have been combined with quarantine

host range studies of native North American butterflies to predict their likely host ranges. This process would almost certainly have made clear the polyphagous natures of *C. glomerata* and *P. puparum*, and their impacts on native butterflies could have been avoided. Currently, host range estimation before the release of new parasitoids is practised by legal mandate in Australia and New Zealand, and by policy in the USA and some other countries. A caution, however, is that methods for host range estimation for parasitoids are just now being developed. In some cases species attacked in the laboratory may escape attack in the field. Benson *et al.* (2003b) found that, even though *P. virginienensis* is within the physiological host range of both *C. glomerata* and *C. rubecula* (Van Driesche *et al.*, 2003), it is unaffected in the field because it is a univoltine woodland species and these parasitoids only forage in meadows. Current practice would not necessarily have predicted this lack of non-target impact.

5. On the whole, this project did a spotty job of assessing whether or not the introduced parasitoids suppressed the target pest in countries where they were released. Most of the evidence that introductions against *P. rapae* lowered the pest's density is contained in Van Driesche and Bellows (1988) or Cameron and Walker (2002). Stronger biological control institutions with long-term funding, a defined mandate and clear rules of conduct should be created to provide for better evaluations of future projects.

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5

Biological Control of the Cassava Green Mite in Africa: Overcoming Challenges to Implementation

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Overview: Ten years after the discovery of the cassava green mite in Africa in the 1970s, the pest had a tremendous negative impact on cassava production across the continent. I address key questions and the remedial actions taken by an international team in the following chapter.

Introduction

The classical biological control campaign against the exotic cassava green mite, *Mononychellus tanajoa*, in Africa has been well documented (Yaninek and Hanna, 2003), but little has been written about the practical challenges that were overcome to implement the programme. For example, how was *M. tanajoa* identified as a suitable candidate for classical biological control? What was known about the taxonomy of *M. tanajoa* and its natural enemies prior to foreign exploration? What biology studies were truly essential when selecting candidate natural enemies and implementing other elements of the biological control campaign? What collaborative expertise and how much cooperation were needed to deliver a programme across a continent? How much did it cost to deliver a classical biological control programme to the poorest continent in the world?

The Campaign

M. tanajoa was discovered feeding on cassava in East Africa in the early 1970s (Fig. 5.1). Presumably, it was introduced accidentally and quickly spread across the African cassava belt, reducing cassava production by a third in its wake (Yaninek *et al.*, 1990). Cassava, a root crop of neotropical origin, is a staple food for a majority of sub-Saharan Africans, so the impact of a new exotic pest like *M. tanajoa* was immediate and the concern real.



Fig. 5.1 Cassava green mite, *Mononychellus tanajoa*, adult female (lower left), adult male (right), and nymphs feeding on the abaxial surface of a cassava leaf.

The campaign to control *M. tanajoa* using biological control was initiated when I was hired in 1983 by the International Institute of Tropical Agriculture to lead the effort (Yaninek and Herren, 1988). Our team launched an extensive foreign exploration effort, and among the more than 50 phytoseiid species found associated with the mite in the neotropics, 11 promising species, and as many or more distinct populations of several species, were shipped to Africa for experimental releases (Yaninek *et al.*, 1993). We also introduced the entomopathogenic fungus *Neozygites tanajoe* (formerly *Neozygites floridana*) from South America as part of this campaign, which I will not discuss further in this chapter. Between 1984 and 2001, more than 11.6 million mass-reared phytoseiids were shipped to 16 countries for release in 847 sites (Table 5.1). Three of these species became established, two spread beyond their original release sites, and one has become established in more than 20 countries, where it has reduced *M. tanajoa* populations and significantly increased cassava yields by a third (Yaninek *et al.*, 1992, 1999; Yaninek and Hanna, 2003). This campaign saved the equivalent of hundreds of millions of dollars in food aid each year. So what were some of the challenges overcome to achieve this success?

Candidate for Biological Control

It was clear from the start that *M. tanajoa* was an exotic pest of cassava in Africa. Like its host plant, *M. tanajoa* was previously known from cassava and related *Manihot* species in the New World. Cassava in the neotropics is relatively tolerant of indigenous pests, particularly locally adapted varieties used in traditional farming systems (Bellotti *et al.*, 1987). In addition, natural enemies of *M. tanajoa*

Table 5.1. Phytoseiid species imported from South America and released and recovered in Africa from 1984 to 2001.

Phytoseiid species	No. countries	No. shipped	No. release sites	Rate of recovery ^a
<i>Amblyseius aerialis</i>	7	142,066	27	0.00
<i>Euseius concordis</i>	8	170,262	44	0.00
<i>Galendromus annectens</i>	10	516,728	41	0.13
<i>Neoseiulus anonymus</i>	10	2,029,983	71	0.30
<i>Neoseiulus californicus</i>	8	443,512	30	0.28
<i>Neoseiulus idaeus</i>	12	5,556,846	241	0.45
<i>Phytoseiulus mexicanus</i>	1	726	1	0.00
<i>Typhlodromalus aripo</i>	16	400,587	220	0.90
<i>Typhlodromalus limonicus</i>	3	144,000	20	0.00
<i>Typhlodromalus manihoti</i>	9	2,130,519	159	0.54
<i>Typhlodromalus tenuiscutus</i>	1	33,452	4	0.00

^aproportion of releases with subsequent recoveries of introduced phytoseiids.

in South America were found to preserve nearly a third of the cassava root production when both the pest and natural enemies were present (Braun *et al.*, 1989). The absence of efficacious natural enemies in Africa that were present in South America made a compelling case for classical biological control.

An early challenge to the programme was the relatively weak support for biological control initiatives of any kind from the international plant protection establishment. This was not surprising given the long history of plant breeding as the focus of the plant protection establishment in Africa (Hahn *et al.*, 1989), and no records of successful classical biological control programmes against spider mite pests like *M. tanajoa* on a subsistence field crop like cassava outside of highly managed greenhouse and orchard production systems. Plant breeders and precedence aside, we moved forward with a classical biological control approach, and in less than 15 years made a mark with one of the most successful biological control programmes ever on a continental scale.

Untangling the Taxonomy

Getting the taxonomy right is a perennial concern for any biological control programme. *M. tanajoa* was a single species until 1981, when *Mononychellus*

progressivus Doreste was described as one of three new species from *M. tanajoa* (Doreste, 1981). Consequently, Gutierrez (1987) identified *M. tanajoa* in Africa as *M. progresivus*, but this soon became controversial when Doreste no longer recognized *M. progresivus* as a good species. Apparently, Doreste based his description on specimens preserved in methanol instead of ethanol, which distorted the integument of the preserved mites, causing their dorsal setae to appear relatively larger than those normally found on *M. tanajoa*. Although the potential implications of the disputed taxonomy on foreign exploration were never clear since both 'species' in South America had potentially wide, overlapping distributions, foreign exploration in this campaign proceeded as if *M. tanajoa* in South America and Africa was the same species.

A bigger taxonomic concern for the campaign was what natural enemies to look for. Predatory mites of the family Phytoseiidae were prime targets right from the start, given their well-known association with spider mites around the world (Yaninek and Moraes, 1991). The problem was the limited knowledge about specific species associated with cassava in South America (Moraes *et al.*, 1986), and virtually a blank slate for species in Africa (Yaninek and Herren, 1988). Our remedy was to survey the common phytoseiid mite predators found associated with cassava in the neotropics and Africa, to serve as a guide in selecting candidate biological control agents and help distinguish introduced species in Africa from the background mite fauna. In Africa, more than 160,500 mite specimens representing 79 genera and 170 species in 33 families were collected from 496 host plant species in 26 countries over a 10-year period (Yaninek and Hanna, 2003). By design, the largest group of specimens belonged to the Phytoseiidae, which were represented by 18 genera and 103 known and 26 undescribed species from 402 host plant species. This led to descriptions of 20 new species and 63 re-described species found in Africa, and 16 new species and 68 re-described species found in South America, which continues today (G. J. Moraes, Piracicaba, Brazil, 2005, personal communication).

Essential Studies

Prior to its discovery in Africa, virtually nothing was known about *M. tanajoa* anywhere in the world (Yaninek and Herren, 1988). Even basic questions about pest biology and ecology had to be addressed locally. Where do you start when you know nothing about a pest except its name and origin? Since this pest was new to Africa, the spread across the cassava belt and the damage it caused to cassava production were our priorities (Yaninek, 1988; Yaninek *et al.*, 1990). Once we established its distribution and agronomic impact, the agroecological conditions that prompted outbreaks and maintained *M. tanajoa* populations at high densities were measured to help identify homologous regions in the neotropics as targets for foreign explorations (Yaninek and Bellotti, 1987).

Systems modelling identified critical interactions in the cassava/mite/predator tritrophic ecosystem and helped us prioritize the research and development agenda for the programme (Gutierrez *et al.*, 1999). This included deliberate laboratory and field studies designed to improve the operational efficiencies of

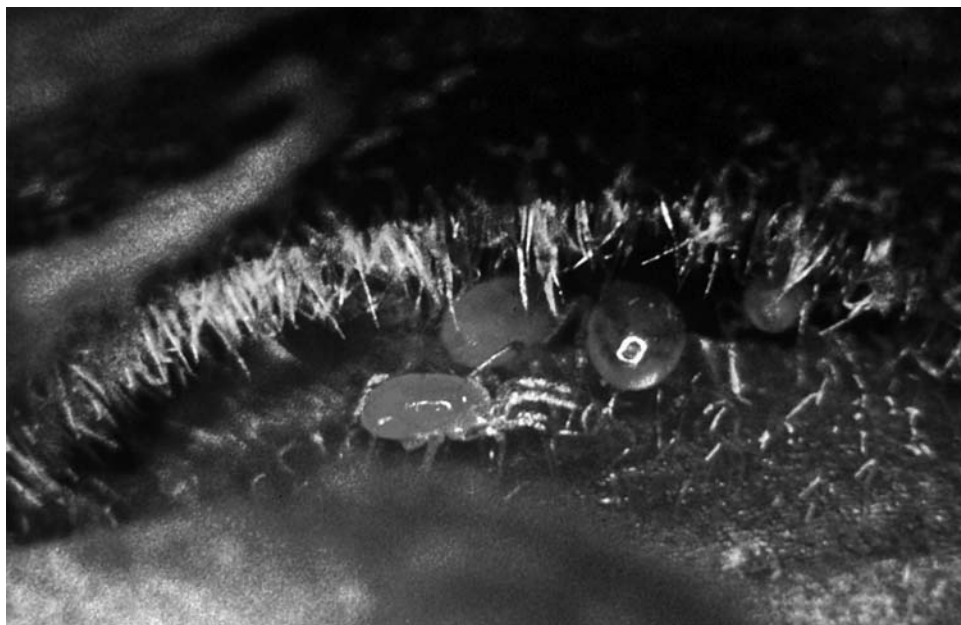


Fig. 5.2. Phytoseiid predators, *Typhlodromalus aripo*, between the folds of the apex of a cassava plant.

many aspects of the implementation programme. We developed new procedures for large-scale mass production of natural enemies, pre-release surveys of cassava green mite incidence/abundance/severity and associated natural enemies, novel and efficient methods to rear, transport and release natural enemies, post-release follow-up monitoring, pest and natural enemy identification, and impact assessment (Megevand *et al.*, 1993).

The campaign team initiated a series of biology studies on introduced phytoseiid predators to establish a basis for evaluating their impact on *M. tanajoa* in Africa. These studies revealed that the least likely predator, *Typhlodromalus aripo*, was the best natural enemy for *M. tanajoa* in Africa (Figs 5.2 and 5.3). This predator, although less voracious and slower in population increase than *Typhlodromalus manihoti* and *Neoseiulus idaeus*, was best at establishment, dispersal and persistence on cassava because it used cassava tips as a refuge (Onzo *et al.*, 2003), efficiently located prey (Gnanvossou *et al.*, 2001), and persisted at low prey densities owing to alternative food sources including pollen and cassava extrafloral exudates (Yaninek and Hanna, 2003).

Selecting Natural Enemies

Ultimately, the success of any classical biological control campaign rests on finding and establishing well-adapted and effective natural enemies. There is a



Fig. 5.3. The phytoseiid predator, *Typhlodromalus aripo*, feeding on a cassava green mite, *Mononychellus tanajoa*, adult female.

considerable body of literature devoted to natural enemy selection that provides informed ecological reading, but not much that can be used in a practical way when searching for candidate natural enemies. The challenge is having the foresight to anticipate the biological interactions following an introduction that ultimately make or break a candidate natural enemy.

Rather than looking for natural enemies exclusively based on a set of theoretical characteristics, our foreign exploration efforts in South America initially focused on regions that were agroclimatic homologues to regions in Africa where *M. tanajoa* outbreaks occurred and high populations persisted on a regular basis (Yaninek and Bellotti, 1987). Within each target region, we looked for the phytoseiid predators found associated with cassava where *M. tanajoa* was present, especially at low population densities, and screened early candidate natural enemies for their ability to feed and reproduce on *M. tanajoa* before being considered for importation (Yaninek *et al.*, 1993).

Collaboration and Training

This campaign could not have been implemented without the contributions of national partners in 20 participating countries in Africa, and international experts from four continents. The campaign foundation was built on a cadre of highly trained and dedicated core staff developed by a small group of biological control specialists recruited specifically for this purpose (Herren, 1989). We recruited and trained local staff to work on basic biology and ecology, natural-enemy mass production and experimental releases, pre- and post-release monitoring, and training. The operational team grew to 35 individuals at the peak of the project. Operational units were supervised by local university graduates with backgrounds in agriculture and biology. Many of these individuals eventually completed PhDs, as part of the programme in most instances.

National collaborators provided the access needed to implement the programme in participating countries. Typically, a key individual was someone with an interest in plant protection who worked for the ministry of agriculture.

These individuals made all the contacts and local arrangements required for the programme, including arranging for the import permits to introduce and monitor biological control agents. Since most national programme staff had no experience working with mites of any kind, we routinely arranged subject matter and leadership training for the primary contacts followed by in-country training of programme support personnel (Yaninek and Schulthess, 1993; Yaninek *et al.*, 1994).

International cooperators provided critical expertise beyond the capacity of project personnel in Africa. This included mite taxonomy, foreign exploration, international quarantine, selected contract research, and MSc and PhD degree training in some cases. Reliable taxonomic expertise is often hard to come by, but is essential in classical biological control projects. We identified plant feeding and predatory mite specialists as essential collaborators early in the campaign. Foreign exploration by definition was beyond the boundaries of Africa and required cooperators with a local knowledge of cassava, *M. tanajoa* and their natural enemies. We contracted for foreign exploration to be undertaken by collaborators at Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) in Brazil, and the Centro Internacional de Agricultura Tropical (CIAT) in Colombia. Natural enemies shipped to Africa for experimental releases first passed through quarantine in Europe (initially Imperial College in England, then later the University of Amsterdam in the Netherlands). Universities in Africa, South America, North America and Europe hosted project staff and cooperators for higher degree training and produced 15 PhD, 9 MSc and 9 BSc degrees.

We developed and delivered training in basic acarology and biological control applications as a core activity for participating national programmes. Hundreds of staff and collaborators received in-service training every year. Similarly, bench training was done for 25 national programme collaborators with specialized needs and implemented through a series of internships. Training created the basis for all subsequent research and development activities in collaborating national programmes and is the main reason why the campaign was successful in most countries.

The Costs

Cassava farmers were not the only resource-poor constituents associated with this campaign. Most national programme staff lacked the training, infrastructure, or experience to participate in a campaign like this without significant operational support. Consequently, the campaign generated an estimated US\$9.5 million to implement the programme from 1983 to 1997 (Table 5.2). We allocated approximately US\$680,000 a year for operations at headquarters, a network of national collaborators that grew to 20 countries, contracted services for foreign exploration, international quarantine and selected research, and training. National programmes needed resources for everything from personnel, basic lab and field equipment, electricity to run the lab, vehicles, fuel, communications, computers, and often the most basic supplies, e.g. pencils and paper. Considering the urgency and scope of the problem, the need for basic infrastructure, and the

Table 5.2. Summary expenditures for core project personnel, operations, foreign exploration, international quarantine, contracts, training and national programmes between 1983 and 1997.

Activity	Cost (US\$)
Core personnel	2,219,052
Operations	617,804
Foreign exploration	2,146,302
International quarantine	1,433,403
Contracts	189,124
Training	442,550
National programmes	2,458,609
Total	9,506,844

economic impact of the campaign, the budget was probably a bargain. This investment created expertise, experiences and infrastructure that subsequently benefited other biological control activities in the participating national programmes that far exceeded all expectations (Yaninek and Schulthess, 1993).

Conclusions

The campaign overcame the most daunting challenges to implementation and achieved a number of significant firsts, including the first mite pest on a field crop distributed across a continent to be successfully controlled using introduced natural enemies. This was achieved in spite of the conventional plant protection wisdom at the time because a strong biological, agronomic and economic case was made for a classical biological control solution, and the international donors agreed to support the programme. Long-term support from the donors maintained the campaign momentum, while the network of collaborators and national programme cooperators delivered the programme in more than 20 countries over an initial period of 15 years. Getting the taxonomy right took some effort, but got the campaign off on the right foot, while the prescriptive biology and ecology studies kept us on the right path. These studies directed foreign exploration and candidate natural enemy selection, and helped us evaluate the impact and mechanisms of control by the introduced biological control agents. A cadre of highly trained and dedicated core project staff provided the technical continuity needed for handling and evaluating introduced phytoseiid predators. National programme participants gained training and experience working with mites, and received support needed for the infrastructure and operations required to participate effectively in the programme. Ultimately, the challenges were overcome and the programme achieved its goal of controlling *M. tanajoa* in Africa using classical biological control. The campaign has had an enormous impact on subsistence agriculture in

Africa and created a legacy of biological control training, infrastructure and experience that is still evident today.

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6

The Multicoloured Asian Ladybird Beetle: Beneficial or Nuisance Organism?

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The road to hell is paved with good intentions.

(Frequently but mistakenly credited to Samuel Johnson: the original quotation should be credited to Saint Bernard of Clairvaux (1091–1153) as 'Hell is full of good intentions or desires.')

Overview: The multicoloured Asian ladybird beetle is one of the most voracious and polyphagous coccinellid predators in the world. It has been introduced in North America as a biocontrol agent to help agriculture. This is the story of several unexpected problems that arose. Because of its polyphagy and aggressiveness as a predator, it displaces several indigenous and imported coccinellid species in several agro-ecosystems. In the autumn, it frequently enters houses by the thousands and is a serious nuisance. In vineyards, adults taint the wine when grapes are pressed at harvest.

Ladybird Beetles: Friend or Foe?

In the minds of most people, coccinellids are friendly and beneficial insects. They are brightly coloured, ubiquitous and are predators that help farmers. When we were young, we read poems and drew pictures of ladybugs while in school. Teachers used coccinellids to illustrate to us basic ecological principles. These beetles are easy to rear and they make excellent experimental subjects to test numerous hypotheses relevant to biocontrol. In college biology courses, we learned that, except for a few phytophagous species, coccinellids are voracious predators and are major players in successful biocontrol programmes. In other words, we have been taught that coccinellids are almost perfect. But are they?

As scientists, we know that things are generally not that simple. A case in point is *Harmonia axyridis* (Coleoptera: Coccinellidae) (Fig. 6.1), the multicoloured Asian lady beetle (MALB) (also named the Harlequin ladybird in England), a coccinellid that was released in the USA for biocontrol purposes (Koch, 2003; Pervez and Omkar, 2006). Why did a biocontrol programme, intended for the public good in agriculture, unexpectedly turn sour, to the point where negative headlines were published in the major media of North America? This was an unusual and unfortunate situation that may have destroyed years of efforts by scientists to demonstrate the value of biological control programmes to the general public.

Why did this happen? What can we learn from this experience? To answer these questions we will first briefly discuss the origin, biology and releases of *H. axyridis*. Then, we will address its intended effects in agricultural systems. Finally, the unintended consequences of this beetle will be discussed from two points of view, agricultural (e.g. intraguild effects and *H. axyridis* as prey) and non-agricultural settings.

A Few Attributes of Coccinellids

Among coccinellids, the palaearctic *H. axyridis* has a high potential for biocontrol. The species is one of the largest aphidophagous coccinellids, is highly voracious, polyphagous (its diet includes aphids, pollen, lepidopteran and coleopteran eggs and larvae, mites and probably any small, soft-bodied insect), and eurytopic (its habitat includes marshes, forest, agricultural and urban areas) (Iablokoff-Khnzorian, 1982). It can be successfully reared on spoiled grapes, is considered the most fecund species of all coccinellids (Iablokoff-Khnzorian, 1982) and adapts quite easily to different environments. Because of these traits, this beetle is a prime candidate for classical biocontrol programmes. Incidentally, it is the



Fig. 6.1. *Harmonia axyridis* adult (photo by Olivier Aubry).

coccinellid species showing the largest diversity of colour variants in the world – a scientifically puzzling feature that makes correct identification by laypersons difficult.

Origin and Dispersion over the Years – from Eastern Asia to World Invasion

The beetle originated in north-eastern Asia (Iablokoff-Khnzorian, 1982). Japanese specimens were released several times in North America as a biological control agent, notably in California in 1916, 1964 and 1965 and in Nova Scotia, Connecticut, Delaware, Georgia, Louisiana, Maine, Maryland, Mississippi, Ohio, Pennsylvania and Washington from 1978 to 1982 (Gordon, 1985).

The first established population was reported in Louisiana in 1988 (Chapin and Brou, 1991) and it spread rapidly across North America (Teddners and Schaefer, 1994; Coderre *et al.*, 1995). It is now distributed from coast to coast, from Florida to the 48th parallel in Quebec. The species was also recently found in South America (de Almeida and da Silva, 2002).

Releases of *H. axyridis* were also conducted in the Ukraine in 1964, south-eastern Kazakhstan in 1969, southern France in 1982, Azores Islands and Greece in 1993, and Argentina at the end of the 1990s. Observations of established populations of this species in Europe were recently reported in Great Britain, southern France, Greece, Germany and Belgium (Adriaens *et al.*, 2003).

H. axyridis as a Biocontrol Agent – Intended Effects

Upon review of 27 studies of *H. axyridis* as a biocontrol agent, it is clear that this coccinellid is an effective predator, feeding on 16 different aphid species and other prey such as mites (Tetranychidae), Coleoptera and Lepidoptera (Table 6.1). Six studies found *H. axyridis* had potential as a biocontrol agent (Table 6.1). Six other cases reported negligible or no impact of this beetle on the targeted prey. However, these studies may have overlooked the impact of *H. axyridis* on other non-target prey. Since non-target prey are rarely taken into account in these types of study, the observable impact on prey populations by the beetle may have been affected by other potential prey.

H. axyridis as a Guild Member

H. axyridis belongs to many numerous ecological guilds (sensu Polis *et al.*, 1989), most notably the aphidophagous predators. This guild is diverse and includes ground beetles, lady beetles, hover flies, cecidomyiids, brown and green lacewings, hemipteran predators, parasitoids and pathogens. The arrival of *H. axyridis* has generated tremendous changes in the guild structures and their dynamics. In a study of eight different crops in Quebec, *H. axyridis* became

Table 6.1. Efficiency of biological control by *Harmonia axyridis* on different pest species in different introduction countries.

Pest species	Plant species	Study	Biological control	Country or states	References
1 <i>Matsucoccus resinosae</i> Bean and Godwin, red pine scale	<i>Pinus resinosa</i> Ait.	Field cages, Field	potential	Connecticut, USA	McClure, 1987
2 <i>Monellia caryella</i> (Fitch) blackmargined aphid	pecan orchard	Field	excellent control	Georgia, USA	Tedders and Schaefer, 1994
<i>Monelliopsis pecanis</i> Bissell, yellow pecan aphid	pecan orchard	Field	excellent control	Georgia, USA	
3 <i>Macrosiphum rosae</i>	roses	Field	effective	France	Ferran <i>et al.</i> , 1996
4 Pecan aphid complex	pecan orchard	Field	effective (IPM)	New Mexico, USA	LaRock and Ellington, 1996
5 <i>Aphis gossypii</i> Glover, cotton aphid	cucumbers	Field	not effective	Switzerland	Fischer and Leger, 1997
6 <i>Phorodon humili</i> (Shrank), damson-hop aphid	hops	Field	effective	France	Trouve <i>et al.</i> , 1997
7 <i>Aphis gossypii</i> Glover, cotton aphid	melon	Field	effective	Italy	Orlandini and Marteluci, 1997
8 <i>Aphis spiraeicola</i> Pagenstecher, spirea aphid	apple orchard	Field	effective	West Virginia, USA	Brown and Miller, 1998
9 Pecan aphid	pecan orchard	Field	effective	southeastern USA	Rice <i>et al.</i> , 1998
10 <i>Toxoptera citricida</i> , brown citrus aphid	citrus groves	Field	effective	Puerto Rico, Florida	Michaud, 1999
11 <i>Adelges tsugae</i>	hemlock tree	Field	negligible	North Carolina, Virginia	Wallace and Hain, 2000
12 <i>Diaphorina citri</i> Kuwayama, Asian citrus psyllid	citrus groves	Field	potential	Florida, USA	Michaud, 2002a

(Continued)

Table 6.1. Continued.

Pest species	Plant species	Study	Biological control	Country or states	References
13 <i>Diaprepes abbreviates</i> (L.), root weevil (Coleoptera: Curculionidae)	citrus	Laboratory, Greenhouse	potential	Florida, USA	Stuart <i>et al.</i> , 2002
14 <i>Tetranychus urticae</i> Koch, twospotted spider mite	apple trees	Laboratory	not effective	Québec, Canada	Lucas <i>et al.</i> , 2002
<i>Aphis citricola</i> Van der Goot, spirea aphid		Laboratory	not effective		
15 <i>Rhopalosiphum maidis</i> , corn aphid	sweetcorn	Field	potential	New York, USA	Musser and Shelton, 2003c
16 <i>Ostrinia nubilalis</i> (Hübner) (Lepidoptera: Crambidae)	sweetcorn	Field and Laboratory	negligible	New York, USA	Musser and Shelton, 2003b
17 <i>Acyrtosiphon pisum</i> (Harris), pea aphid	lucerne	Field and Laboratory	additive	Wisconsin, USA	Snyder and Ives, 2003
18 <i>Diaphorina citri</i> Kuwayama, Asian citrus psyllid	citrus groves	Field	effective	Florida, USA	Michaud, 2004
19 <i>Aphis glycines</i> Matsumura, soybean aphid	soybean	Field	effective	Michigan, USA	Fox <i>et al.</i> , 2004
20 <i>Aphis spiraecola</i> , spirea aphid	apple orchard	Field	effective	West Virginia, USA	Brown, 2004
21 <i>Macrosiphum euphorbiae</i> Thomas, potato aphid	roses	Greenhouse	complementary	Washington, USA	Snyder <i>et al.</i> , 2004a
22 <i>Aphis gossypii</i> Glover, cotton aphid	cotton	Laboratory	potential	UK	Tsaganou <i>et al.</i> , 2004

23	<i>Leptinotarsa decemlineata</i> , Colorado potato beetle (Coleoptera: Chrysomelidae)	potato	Laboratory	no change	Washington, USA	Snyder and Clevenger, 2004
24	<i>Paraprociophilus tessellatus</i> (Fitch), woolly alder aphid	<i>Alnus serrulata</i> (Ait.)	Laboratory	preference	eastern USA	Butin <i>et al.</i> , 2004
25	<i>Macrosiphum</i> <i>euphorbiae</i> Thomas, potato aphid	potato crops	Field	effective	northern Maine, USA	Alyokhin and Sewell, 2004
	<i>Aphis nasturtii</i> Kaltenbach, buckthorn aphid		Field	effective		
	<i>Myzus persicae</i> Sulzer, green peach aphid		Field	effective		
26	<i>Panonychus citri</i> (McGregor), citrus red mite	citrus	Laboratory	potential	Florida, USA	Villanueva <i>et al.</i> , 2004
27	<i>Rhopalosiphum maidis</i> , corn aphid	maize	Field cages	effective	Québec, Canada	Labrie <i>et al.</i> , unpubl. data

Note: 1. *Environmental Entomology* 16, 224–230; 2. *Entomological News* 105, 228–243; 3. *European Journal of Entomology* 93, 59–67; 4. *Southwestern Entomologist* 21, 153–166; 5. *Revue Suisse de Viticulture, Arboriculture et Horticulture* 29, 119–126; 6. *Entomophaga* 42, 57–62; 7. *Colture Protette* 6, 33–36; 8. *Entomological News* 109, 143–151; 9. *American Journal of Alternative Agriculture* 13, 111–123; 10. *BioControl* 44, 347–367; 11. *Environmental Entomology* 29, 638–644; 12. *Entomological News* 113, 216–222; 13. *Florida Entomologist* 85, 409–416; 14. *European Journal of Entomology* 99, 457–463; 15. *Journal of Economic Entomology* 96, 71–80; 16. *Environmental Entomology* 32, 1131–1138; 17. *Ecology* 84, 91–107; 18. *Biological Control* 29, 260–269; 19. *Environmental Entomology* 33, 608–618; 20. *Biological Control* 29, 189–198; 21. *Biological Control* 30, 229–235; 22. *Biological Control* 31, 138–144; 23. *Biological Control* 31, 353–361; 24. *Journal of Economic Entomology* 97, 1635–1641; 25. *Biological Invasions* 6, 463–471; 26. *Journal of Entomological Science* 39, 23–29.

the most commonly observed species of the coccinellid assemblage in all cases, only a few years after its arrival.

In addition, the introduction of generalist entomophagous insects can have a drastic impact on native competing species (Simberloff and Stiling, 1996; van Lenteren *et al.*, 2003; Kimberling, 2004). It appears that *H. axyridis* impacts its competitors by exploitative competition and/or by intraguild predation (Table 6.2). In the 24 studies on impact of *H. axyridis* on non-target species, 15 aphidophagous species, including 11 coccinellids, were identified as intraguild prey and two other species were non-target insects that could be vulnerable (Table 6.2). *H. axyridis* was also observed eating the chrysomelid *Galerucella calmariensis*, which is a biocontrol agent of the plant *Lythrum salicaria* (Table 6.2). *H. axyridis* can also be a potential prey of other guild members, with most intraguild predation occurring during the early instars. However, after the third instar, *H. axyridis* seems to be out of danger. The time of arrival of *H. axyridis* and its competitors at the aphid colony also determines the relative size of the aphidophagous organisms and consequently the occurrence, direction and symmetry of the intraguild predation between *H. axyridis* and its competitors (Lucas, 2005). In summary, *H. axyridis* can have a significant impact upon guilds it belongs to. It is often the intraguild predator and rarely the intraguild prey.

***H. axyridis* as a Phytophagous Species**

H. axyridis exploits an array of animal (zoophagous) resources, but also may exploit plant material (phytophagous) such as pollen and nectar. Recently, *H. axyridis* has been documented to feed on fruit in the autumn, mainly when they are injured (Table 6.3). This damage can be significant, and the beetle has attained pest status in some fruit crops when its populations explode. In vineyards, grapes can be tainted by the alkaloids that are released by the beetles when they are crushed with the grapes before the fermentation process (Ejbich, 2003). Other fruit attacked in the autumn include plums, raspberries and apples (Table 6.3).

***H. axyridis* as a Food Source**

In ecosystems, high-order natural enemies may benefit from the establishment of a new introduced species if this new abundant biomass can be exploited. However, *H. axyridis* appears to have few natural enemies in habitats where it has been introduced. Although several parasitoids have been reported to attack *H. axyridis*, none are considered effective in managing populations (Katsoyannos and Aliniaze, 1998; Firlej *et al.*, 2005).

Disease organisms such as the entomopathogenic fungus *Beauveria bassiana* seem not to infect *H. axyridis* (Cottrell and Shapiro-Ilan, 2003). Observations in the palaearctic zone indicated that eight bird species effectively prey on this coccinellid, but they exert limited control (Netshayev and Kuznetsov,

Table 6.2. Non-target impacts of *H. axyridis* on coccinellid species, other intraguild prey and other insects.

Non-target species	Plant species	Effect	Country or states	References
Coccinellid species				
1 <i>Coccinella septempunctata</i>	Apple orchard	Abundance decrease	West Virginia, USA	Brown and Miller, 1998
2 <i>Coleomegilla maculata</i> DeGeer	Laboratory	IGP	Kentucky, USA	Cottrell and Yeargan, 1998
3 <i>Brachiacantha ursina</i> <i>Cycloneda munda</i> <i>Cycloneda sanguinea</i> L.	Agricultural landscape	Abundance decrease Abundance decrease Abundance decrease	Michigan, USA	Colunga-Garcia and Gage, 1998
4 <i>Adalia bipunctata</i> L.	Laboratory	IGP; possible impact in field	Japan	Kajita <i>et al.</i> , 2000
5 <i>Adalia bipunctata</i> L.	Laboratory	IGP	Italy	Burgio <i>et al.</i> , 2002
6 <i>Cycloneda sanguinea</i> L.	Citrus groves	Abundance decrease	Florida, USA	Michaud, 2002b
7 <i>Coccinella septempunctata</i>	Apple orchard	Abundance decrease	West Virginia, USA	Brown, 2003
8 <i>Adalia bipunctata</i> L.	Laboratory	Negligible impact	Italy	Santi <i>et al.</i> , 2003
9 <i>Coleomegilla maculata</i> DeGeer	Field, sweetcorn	IGP	New York, USA	Musser and Shelton, 2003a
10 <i>Coccinella transversoguttata</i> Brown	Potato crop	Abundance decrease	Maine, USA	Alyokhin and Sewell, 2004
11 <i>Adalia bipunctata</i> L.	Laboratory	Possible impact in field	Norwich, UK	Sato and Dixon, 2004
12 <i>Coccinella transversoguttata</i> Brown	Laboratory	IGP	Washington, USA	Snyder <i>et al.</i> , 2004b
<i>Hippodamia convergens</i> Guérin-Ménéville	Laboratory	IGP	Washington, USA	
13 <i>Coleomegilla maculata</i> DeGeer	Laboratory	Possible impact in field	Kentucky, USA	Cottrell, 2004
<i>Olla v-nigrum</i> Mulsant	Laboratory	Possible impact in field	Kentucky, USA	

(Continued)

Table 6.2. *Continued.*

Non-target species	Plant species	Effect	Country or states	References
14 <i>Coleomegilla maculata lengi</i> Timberlake	Field cage, maize	Abundance decrease, high resource	Québec, Canada	Labrie <i>et al.</i> , unpublished data
15 <i>Coleomegilla maculata lengi</i> Timberlake	Laboratory	IGP	Québec, Canada	Labrie <i>et al.</i> , unpublished data
<i>Propylea</i> <i>quatuordecimpunctata</i> L.	Laboratory	IGP	Québec, Canada	
16 <i>Coccinella undecimpunctata</i> L.	Laboratory	IGP	Azores, Portugal	Félix and Soares, 2004
17 <i>Coccinella transversoguttata</i> Brown	Laboratory	IGP	Utah, USA	Yasuda <i>et al.</i> , 2004
<i>Hippodamia convergens</i> Guérin-Méneville	Laboratory	IGP	Utah, USA	
18 <i>Coleomegilla maculata</i> DeGeer	Laboratory	IGP	Georgia, USA	Cottrell, 2005
<i>Olla v-nigrum</i> Mulsant	Laboratory	IGP		
Other intraguild prey				
19 <i>Chrysoperla carnea</i> Stephens (Neuroptera: Chrysopidae)	Laboratory	IGP	Iowa, USA	Phoofolo and Obrycki, 1998
20 <i>Aphidoletes aphidimyza</i> Rondani (Diptera: Cecidomyiidae)	Field	Abundance decrease	West Virginia, USA	Brown, 1999

21	<i>Aphidius ervi</i> Halyday (Hymenoptera: Braconidae)	Field and laboratory, lucerne	IGP	Wisconsin, USA	Snyder and Ives, 2003
22	<i>Tamarixia radiata</i> (Waterston) (Hymenoptera: Eulophidae)	Citrus groves	IGP	Florida, USA	Michaud, 2004
Other insects					
23	<i>Danaus plexippus</i> L. (Lepidoptera: Nymphalidae)	Laboratory and field cages	Abundance decrease	Minnesota, USA	Koch <i>et al.</i> , 2003; Koch <i>et al.</i> , 2005
24	<i>Galerucella californiensis</i> L. (Coleoptera: Chrysomelidae)	<i>Lythrum salicaria</i> , field and laboratory	Predation; Potential disruption of biocontrol of <i>Lythrum salicaria</i>	Michigan, USA	Sebolt and Landis, 2004

Note: 1. *Entomological News* 109, 143–151; 2. *Journal of Kansas Entomological Society* 71, 159–163; 3. *Environmental Entomology* 27, 1574–1580; 4. *Applied Entomology and Zoology* 35, 473–479; 5. *Biological Control* 24, 110–116; 6. *Environmental Entomology* 31, 827–835; 7. *BioControl* 48, 141–153; 8. *Bulletin of Insectology* 56, 207–210; 9. *Environmental Entomology* 32, 575–585; 10. *Biological Invasions* 6, 463–471; 11. *Agricultural and Forest Entomology* 6, 21–24; 12. *Oecologia* 140, 559–565; 13. *Biological Control* 31, 362–371; 16. *European Journal of Entomology* 101, 237–242; 17. *Oecologia* 141, 722–731; 18. *Biological Control* 34, 159–164; 19. *Entomologia Experimentalis et Applicata* 89, 47–55; 20. *IOBC/wprs Bulletin* 22, 7; 21. *Ecology* 84, 91–107; 22. *Biological Control* 29, 260–269; 23. *Biological Control* 28, 265–270; *Environmental Entomology* 34, 410–416; 24. *Environmental Entomology* 33, 356–361.

Table 6.3. Negative impacts of *H. axyridis* on plants.

Plant attacked	Country or state	References
1 Apples	Minnesota, USA	Hutchison <i>et al.</i> , 2003
2	Minnesota, USA	Koch <i>et al.</i> , 2004
3	Ohio, USA	Kovach, 2004
4 Grapes	North central USA	Ratcliff, 2002
5	Ohio, USA	Williams <i>et al.</i> , 2002
6	Ontario, Canada; northern USA	Ejbich, 2003
7	Minnesota, USA	Hutchison <i>et al.</i> , 2003
8	Minnesota, USA	Koch <i>et al.</i> , 2004
9	Ohio, USA	Kovach, 2004
10	Ontario, Canada; Ohio, USA	Pickering <i>et al.</i> , 2004
11 Peaches	Ohio, USA	Kovach, 2004
12 Plums	Québec, Canada	M. Roy, pers. comm.
13 Pumpkins	Minnesota, USA	Koch <i>et al.</i> , 2004
14 Raspberries	Minnesota, USA	Hutchison <i>et al.</i> , 2003
15	Minnesota, USA	Koch <i>et al.</i> , 2004

Note: 1. *Minnesota IPM Vegetable Newsletter* 5; 2. *Journal of Economic Entomology* 97, 539–544; 3. *American Entomologist* 50, 159–161; 4. USDA CSREES Regional Integrated Pest Management Program and the Pest Management Centers, 1–2; 5. *Arthropod Management Tests* 27, L14; 6. *Wine Spectator* 15 May, 16; 7. same as 1; 8. same as 2; 9. same as 3; 10. *American Journal of Enology and Viticulture* 55, 153–159; 11. same as 3; 13. same as 2; 14. same as 1; 15. same as 2.

1973 in Hodek and Honek, 1996). No experiments have been carried out in North America to evaluate mortality caused by birds.

Reading these studies results in two conclusions. First, *H. axyridis* seems relatively enemy-free in the invaded countries and, second, higher-order enemy species are unable to successfully exploit this lady beetle as a food source.

***H. axyridis* as a Public Nuisance**

In eastern North America during the mid-1990s, we began receiving reports from homeowners that ladybugs were infesting their houses. At first, it was difficult to believe that people were complaining about a few lady beetles in their homes (don't they realize that they are beneficial?) but when thousands were observed in their houses year after year, we knew we had a problem. In the autumn, adult ladybird beetles enter houses through cracks and crevices. Homeowners reported that during the swarming period (in the autumn), they could not leave their houses without being covered with beetles, which frequently bit them (Huelsman and Kovach, 2004; Kovach, 2004). When present in large numbers, *H. axyridis* aggravate homeowners by making their way into food and drinks, and disrupting activities such as sleeping and reading. In addition, when

the beetles are disturbed, they release a yellow-orange, foul-smelling liquid that stains many surfaces and clothing (Huelsman and Kovach, 2004; Kovach, 2004). Also, they can be a nuisance in hospitals and testing facilities of pharmaceutical companies, where there is zero tolerance for biocontaminants (Nalepa *et al.*, 2005).

H. axyridis is becoming a nuisance in Europe as well, where first observations in houses were recorded in Belgium during autumn 2004 (San Martin *et al.*, 2005). Usually *H. axyridis* invades the same sites each year, which lowers the property value of the targeted houses. Because of this overwintering behaviour, *H. axyridis* is now considered a public nuisance (Table 6.4).

Harmonia axyridis can induce allergic reactions in some people. Yarbrough *et al.* (1999) were the first to document an allergy caused by a member of the coccinellid family or with an insect used as a biocontrol agent. In an Ohio survey, up to 26% of homeowners reported some allergic reaction when they were living with large infestations of *H. axyridis* (Hueslman and Kovach, 2004). Observations of *H. axyridis* invading beehives were also reported (Table 6.4).

What Have We Learned from Our Experience with *H. axyridis*?

In classical biological control programmes, specialist and generalist biocontrol agents are compared according to their efficacy and innocuity. It is now recognized that generalist biocontrol agents have a lower success rate in classical biological control programmes and a higher probability to generate non-target effects (van Lenteren *et al.*, 2003; Kimberling, 2004). As illustrated by the generalist biocontrol agent *H. axyridis*, non-target impacts can be surprising and the situation can quickly get out of control. It also highlights the fact that insects have no frontiers (the beetle reached relatively northern latitudes of Canada in just a few years) and that agents often spread to distant areas where they are unwanted (Simberloff *et al.*, 2005). It is impossible to evaluate the overall biological and economic impact of this lady beetle, now and in the future. This case could, however, be a key tool in identifying characteristics that need to be evaluated in a cost-benefit analysis and risk assessment procedures prior to the introduction of new biocontrol agents (Perrings *et al.*, 2005; Simberloff, 2005; Colautti *et al.*, 2006).

The Final Word

Before the arrival of *H. axyridis* in North America, natural and agricultural systems suffered from the arrival of at least three other exotic lady beetles (*Coccinella septempunctata*, *Propylea quatuordecimpunctata* and *Hippodamia variegata*). Composition of the guilds has been impacted by these successive waves of immigrants, but was only of concern to entomologists and ecologists. As pointed out by Elliott *et al.* (1996) for the release of *C. septempunctata*, it is essential to weigh the potential benefits of the release of a polyphagous predator such as *C. septempunctata* or *H. axyridis*. The story of *H. axyridis* should serve

Table 6.4. Negative impacts of *H. axyridis* on humans.

Action	Negative impact	Country or states	References
1 overwintering in beehives	nuisance to beekeepers	Delaware, USA	Caron, 1996
2 overwintering in boxes at railroad crossings	prevent upward movement of the signal alarm	Florida, USA	Mizell, 2002
3 overwintering in houses	allergic rhinoconjunctivitis	USA	Yarbrough <i>et al.</i> , 1999
4	bite	Ohio, USA	Huelsman <i>et al.</i> , 2001
5		Ohio, USA	Kovach, 2004
6	nuisance	Georgia, USA	Tedders and Schaeffer, 1994
7		North Carolina, Virginia, USA	Kidd and Nalepa, 1995
8		North Carolina, Virginia, USA	Nalepa <i>et al.</i> , 1996
9		Oregon, USA	LaMana and Miller, 1996
10		Ontario, Canada	Hagley, 1999
11		Belgium	Adriaens <i>et al.</i> , 2003
12		Pennsylvania, USA	Riddick <i>et al.</i> , 2000; 2004
13		Belgium	San Martin <i>et al.</i> , 2005
14		Québec, Canada	Labrie <i>et al.</i> , unpublished data
15	odour	Ohio, USA	Huelsman <i>et al.</i> , 2001
16	stain on walls	Ohio, USA	Huelsman <i>et al.</i> , 2001
17		Belgium	San Martin <i>et al.</i> , 2005

Note: 1. *American Bee Journal* 136, 728–729; 2. *Florida Entomological Society*, 85 Annual Meeting, July 28–31, Clearwater Beach, Florida, USA; 3. *The Journal of Allergy and Clinical Immunology* 104, 704–705; 4. <http://ipm.osu.edu/lady/icup.htm>; 5. *American Entomologist* 50, 159–161; 6. *Entomological News* 105, 228–243; 7. *Proceedings of the Entomological Society of Washington* 97, 729–731; 8. *Annals of the Entomological Society of America* 89, 681–685; 9. *Biological Control* 6, 232–237; 10. *Publication 208 Agriculture and Agri-Food Canada and Ontario Ministry of Agriculture, Food and Rural Affairs*; 11. *Belgium Journal of Zoology* 133, 195–196; 12. *Annals of the Entomological Society of America* 93, 1314–1321; *Journal of Entomological Science* 39, 373–386; 13. *Insectes* 136, 7–11; 15. same as 4; 16. same as 4; 17. same as 13.

as a wake-up call to all biocontrol specialists and should constitute a lesson for researchers, civil servants and all persons involved in the programmes. This was an example of a human action with huge economic and ethical consequences. Learning from this experience may allow us to select and adopt a legislative framework for the future.

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7

Introduction of a Fungus into North America for Control of Gypsy Moth

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Overview: Soon after its accidental introduction into North America, the gypsy moth started its spread as an alien invasive species, causing severe defoliation of deciduous forests and shade trees. This is the story of the miraculous appearance of an entomopathogenic fungus, which, soon after its appearance, started to decimate gypsy moth populations. It provides a good example of the 'enemy release hypothesis', because high populations of this pest are not common in its areas of origin but severe outbreaks occur in North America where gypsy moth has invaded without its native natural enemies. Many natural enemies were introduced, but after an entomopathogenic fungus became established, gypsy moth populations have remained at much lower densities in many areas.

The Gypsy Moth and Biological Control

In the mid-1800s an entrepreneur named Leopold Trouvelot brought the gypsy moth, *Lymantria dispar* (Fig. 7.1), to the USA from France for hybridization with native North American silkworms, to try to create a stronger silkworm. At that time, silk production was plagued with problems due to a disease that was decimating silkworm colonies. History was made in 1868 or 1869 when some gypsy moths escaped through a broken window in Leopold's house in Medford, Massachusetts. After the escape, gypsy moths slowly began spreading and, as populations moved into new areas, they often increased to outbreak levels, causing extensive defoliation. Over the years since the initial introduction, large federal and state programmes have been implemented to stop the spread of gypsy moth. Gypsy moth has always eventually continued its spread to the west, south and north, although the present control programme is significantly slowing the spread. Since the introduction of gypsy moth to North America, prolonged periods of defoliating populations have occasionally occurred, but gypsy moth is mostly known as an outbreak insect with population eruptions interspersed with long periods of low density. Gypsy moth outbreaks defoliate large areas of urban and suburban



Fig. 7.1. A gypsy moth caterpillar, *Lymantria dispar* (Photo by Tana Ebaugh).

forest in June (700–50,000 km² annually; Montgomery and Wallner, 1988) (Fig. 7.2), leading to decreased tree growth and some tree mortality, along with creating a nuisance. Between 1985 and 2003, federal and state governments spent over \$266 million on the management of gypsy moth outbreaks and slowing the spread of gypsy moth along the invasion front (P.S. Tobin and A.E. Liebhold, personal communication). Thus, gypsy moth provides a good example of the ‘enemy release hypothesis’, because high populations of this pest are not common in its areas of origin, which include Europe, northern Africa and temperate Asia but outbreaks have occurred in North America where gypsy moth has invaded without its native natural enemies.

In 1905, classical biological control programmes were begun to introduce the natural enemies of gypsy moth that control populations in its areas of origin. Most of the gypsy moth natural enemy species introduced have been parasitoids from Europe and Asia. However, in 1908, samples sent from Japan included cadavers of gypsy moth larvae that had been killed by an entomophthoralean fungus. In 1909, G.P. Clinton travelled to Japan, collected some infected gypsy moth larvae near Tokyo and succeeded in bringing two cadavers containing spores of the pathogen back to Harvard University. A.T. Speare and R.H. Colley studied this entomophthoralean fungus and released it in the Boston area in 1910 and 1911. However, by 1912, they briefly summarized their work, stating that their extensive releases had never resulted in establishment of this fungal pathogen, which they referred to as the ‘gypsy fungus’ (Speare and Colley, 1912).



Fig. 7.2. Severe defoliation caused by the caterpillars.

The classical biological control programme continued and, by 1976, the introduction programme against gypsy moth was described as ‘one of the few massive projects in biological control history’ (Hoy, 1976). As has often been typical of classical biological control, results from introductions during this programme were not very predictable, and more introductions and harder work did not always result in establishment. By 1997, after introduction of more than 55 species, one species of predatory beetle and 12 species of parasitic wasps and flies had become established, along with a viral pathogen that had been accidentally introduced (Table 7.1) (Fuester and Taylor, 1997). However, none of these introduced natural enemies provided effective control.

Perhaps the most effective introduced natural enemy before 1989 was the *L. dispar* nucleopolyhedrovirus (LdMNPV), which was first reported in North America in 1907, and probably had been imported and accidentally released along with early releases of parasitoids¹. LdMNPV was relatively predictable in causing precipitous collapses of outbreak populations, but collapses characteristically only occurred after an area had been defoliated for at least 1 year. Larval deaths due to LdMNPV were quite obvious because the highest levels of mortality occurred in late instars and, characteristically, the flaccid bodies of late instars were left hanging on tree trunks, attached by a proleg, eventually breaking to release viral particles into the environment. One scientist working in biological control in 1974 summarized the gypsy moth classical biological control programme saying ‘. . . biological control of the gypsy moth has been completely tried and is hopeless’ (DeBach, 1974), although others felt that the insect natural enemies released had given appreciable control, reducing gypsy moth outbreak range and severity (see Hoy, 1976). Foretelling the future, DeBach (1974) also stated that ‘The question persists if among the remaining twenty-nine natural enemy species not established, or others not yet discovered, the ultimate successful solution lies hidden.’

Table 7.1. Introduced natural enemies of gypsy moth successfully established in North America (Fuester and Taylor, 1997; A.E. Hajek, unpublished data).

Group	Group	Species	Stage attacked	Status
Virus	Baculoviridae	<i>Lymantria dispar</i> nucleopolyhedrovirus (LdMNPV)	All larval instars	Widespread, important in dense populations
Fungi	Fungi: Entomophthorales	<i>Entomophaga maimaiga</i>	All larval instars	Widespread, important in high- and low-density populations
Insecta	Coleoptera: Carabidae	<i>Calosoma sycophanta</i>	Large larvae and pupae	Behind leading edge, in dense populations
	Diptera: Tachinidae	<i>Blepharipa pratensis</i>	Large larvae	Widespread, but declining
		<i>Compsilura concinnata</i>	Large larvae	Widespread, best at low densities
		<i>Exorista larvarum</i>	Large larvae	Rare
	Hymenoptera: Eupelmidae	<i>Parasetigena silvestris</i>	Large larvae	Widespread, important in declining populations
		<i>Anastatus disparis</i>	Egg	Subdominant
		<i>Ooencyrtus kuvanae</i>	Egg	Destroys ca. 1/3 of cohort
	Hymenoptera: Encyrtidae	<i>Monodontomerus aereus</i>	Pupae	Hyperparasitoid of braconids and tachinids
	Hymenoptera: Torymidae			
	Hymenoptera: Chalcididae	<i>Brachymeria intermedia</i>	Pupae	Widespread, important in dense populations
	Hymenoptera: Braconidae	<i>Aleiodes indiscretus</i>	Small larvae	Rare
		<i>Cotesia melanoscela</i>	Small larvae	Widespread, sometimes important
	Hymenoptera: Ichneumonidae	<i>Coccygomimus disparis</i>	Pupae	Widespread, parasitism low
		<i>Phobocampe uncinata</i>	Small larvae	Localized, usually rare

***Entomophaga maimaiga* Becomes Established in North America**

In mid-June 1989, Ted Andreadis of the Connecticut Agricultural Experiment Station in New Haven and Mike McManus of the USDA, Forest Service in Hamden, Connecticut started seeing gypsy moth cadavers hanging on trees (Fig. 7.3). Both of these scientists had been working on gypsy moth for many years and they knew that something was a bit unusual because the gypsy moth population in their area was at low density. One would only expect to see lots of cadavers hanging on tree trunks if the gypsy moth population had been very dense, at which point one would expect to observe abundant mortality due to LdMNPV. Back in the laboratory, microscopic examination of cadavers revealed large resting spores characteristic of entomophthoralean fungi (Fig. 7.4). As more cadavers accumulated on tree trunks, I was contacted because I had been involved with studies of the fungal species infecting gypsy moth in Japan.

In the early 1980s, collections of the Japanese gypsy moth fungus had tempted Dick Soper of the USDA, Agricultural Research Service in Ithaca, New York, and he decided to try, once again, to introduce this fungus to the gypsy moth populations in the USA. In 1984, he travelled with a Japanese scientist, Mitsuaki Shimazu, to the western coast of Honshu, and they successfully found and isolated this obligate fungal pathogen. Isolates were brought back to the Boyce Thompson Institute in Ithaca, New York, where Rich Humber determined that this fungus belonged in the genus *Entomophaga* in the fungal order Entomophthorales and, based on molecular studies and geographic distribution, this fungus was given the species name *maimaiga*, which means ‘gypsy moth’ in Japanese. We made small-scale releases of laboratory-colony larvae injected



Fig. 7.3. Cadavers of late instar gypsy moth larvae killed by *Entomophaga maimaiga* (Photo by Gary Bernon).

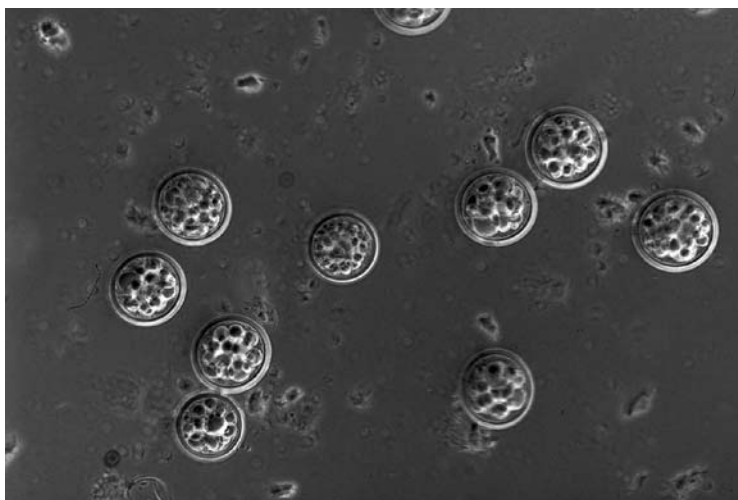


Fig. 7.4. Resting spores of *Entomophaga maimaiga*.

with fungal cells in south-western New York State (1985) and Shenandoah National Park, Virginia (1986) (Hajek *et al.*, 1995b). Gypsy moth had only recently invaded both of these areas and populations were thought to be increasing. Although this fungus grew well in culture and could infect in the laboratory, the field releases were not considered successful because virtually no transmission to the wild population was detected.

The fungus found in 1989 appeared to be the same species as the fungus released in 1910, 1911, 1985 and 1986. Surveys conducted quickly in 1989 demonstrated that the fungus was present in seven north-eastern states, but by 1990 it had been documented in ten states and by 1992 *E. maimaiga* had spread across the contiguous distribution of the gypsy moth in the north-eastern USA. Since that time, spread by *E. maimaiga* has followed behind the leading edge of gypsy moth spread. This fungus spreads on its own through aerial dispersal of actively ejected asexual spores from cadavers of gypsy moth larvae it has killed, but it can also be moved unwittingly by humans. Two different models of spread have suggested that short-range and long-range dispersal both occur, with different characteristics.

E. maimaiga can cause high levels of infection in both high- and low-density populations of gypsy moth, leading to population crashes. Our long-term plots in central New York have documented the re-occurrence of high levels of infection within low-density gypsy moth populations since 1992, when an epizootic (= high level of infection) occurred, resulting in a population crash, through 2006; thereafter, gypsy moth populations have not increased again in central New York, and throughout this time *E. maimaiga* has been the most common natural enemy. Elsewhere in the gypsy moth distribution, *E. maimaiga* has also caused high levels of mortality, leading to reduced gypsy moth populations, and sometimes, when *E. maimaiga* has been very active, this fungal pathogen has prevented defoliation and increase of gypsy moth populations to outbreak densities.

E. maimaiga persists in the top layers of soil as resting spores, which have been shown to persist for at least 11 or 12 years and probably longer. A limiting factor for activity of this fungus can be rainfall during the time when larvae are present (usually May and June); during exceptionally dry springs *E. maimaiga* is less active and therefore infects fewer larvae. Models have suggested that spring-time weather conditions in parts of the mid-western USA might not offer conditions necessary for *E. maimaiga* epizootics as regularly as in the north-eastern USA.

E. maimaiga principally interacts indirectly with other established natural enemies by competing for hosts. For example, *E. maimaiga* and LdMNPV have both been recorded during epizootics at the same sites and even occur within the same hosts. However, if these two pathogens infect the same host, in many cases only *E. maimaiga* will reproduce because the fungal pathogen develops more quickly than the viral pathogen (Malakar *et al.*, 1999). Parasitoids are regularly reared from non-infected gypsy moth larvae collected in long-term plots in central New York where *E. maimaiga* is active each year.

Where Did *E. maimaiga* Presently in North America Come From?

This question has been asked during virtually every presentation I've made about this fungus. *E. maimaiga* occurs in Japan, Korea, north-eastern China and the Russian Far East. Based on molecular studies, we now know that the fungal strain present in North America originated from Japan (Nielsen *et al.*, 2005).

Could this fungus have been successfully introduced by Speare and Colley in 1910–1911? This does not seem likely because the fungal strains established in North America today are quite different from isolates of *E. maimaiga* collected near Tokyo. Also, it seems unlikely that this fungus would not have been found infecting gypsy moth larvae between 1911 and 1989, especially because appropriate weather conditions for epizootics occurred several times during this time-span (Weseloh, 1998) and massive field surveys of gypsy moth pathogens were undertaken. Could this fungus have been successfully introduced in 1985–1986? Once again, molecular studies showed that the isolate that was released was more different from the isolate recovered in 1989 than would have been expected, given that changes in the released isolate would have to have occurred between release of the fungus in 1985–1986 and isolation of strains during and after the 1989 epizootics. At present, the best-accepted explanation for the origin of *E. maimaiga* present today in North America is that an accidental introduction must have occurred at some time since 1971 (Weseloh, 1998).

Studies of Effects of *E. maimaiga* on Non-target Lepidoptera

The gypsy moth and its natural enemies live in naturally occurring forests as well as urban forests. When this virulent pathogen was discovered in North America in 1989 and people began discussing redistribution and potential use for inundative

or inoculative augmentation, questions about environmental safety were posed: Would *E. maimaiga* have unexpected negative impacts on the native fauna? We conducted studies to address this question from 1992 to 2001. We quickly learned that *E. maimaiga* could only potentially affect lepidopteran larvae that are present in the spring when gypsy moth larvae are also present. First, larvae of representatives of the many species of forest Lepidoptera present in the forest during spring at the same time as gypsy moth larvae were collected and challenged with the fungus in the laboratory. Under these optimal conditions *E. maimaiga* could infect about one-third of the 78 species challenged, but infection was only consistently high among the three species of tussock moths (Lymantriidae) tested plus one laboratory colony of a hawk moth (Sphingidae) (Hajek *et al.*, 1995a). We were surprised by these results because in the forest we virtually never saw cadavers of any species other than gypsy moth hanging on tree trunks during epizootics. Therefore, next we collected native lepidopteran larvae in forests in Virginia, Michigan and New York during spring. While some of the gypsy moth larvae collected were always found to be infected, only two of 1511 larvae belonging to 52 species of non-targets from moderate-density gypsy moth sites in Virginia were infected and both of these individuals belonged to relatively common species (Hajek *et al.*, 1996). These results clearly showed that the range of hosts that this natural enemy can infect in the laboratory (the physiological host range) was not equivalent to the range of species infected in the field (the ecological host range).

Meanwhile, during other studies we had found high levels of infection among gypsy moth larvae caged on the soil. We had not investigated the potential for infections in non-target Lepidoptera at the soil surface. Late-instar gypsy moth larvae often spend the daytime hours resting in the leaf litter, and our studies have shown that this behaviour, which is highly unusual for most lepidopteran larvae, results in high levels of infection (Hajek, 2001). Studies of lepidopteran larvae in the leaf litter yielded extremely low levels of infection except among gypsy moth larvae (Hajek *et al.*, 2000).

The only non-target Lepidoptera consistently infected in the laboratory were lymantriids, which belong to the same family as gypsy moth. No lymantriids occurred in the leaf litter during our studies and few had been collected during our foliage studies. Therefore, we needed to focus our efforts on larvae of this family of endemics, which are often less common. Over 5 years, all lymantriids present in 18 plots in the forested mountains of Virginia and West Virginia were reared to detect infections (Hajek *et al.*, 2004). Among the seven species of native lymantriids collected, only three were ever found to be infected by *E. maimaiga* and never at 50%, although infection among gypsy moth larvae was high. In summary, our extensive studies have demonstrated that this obligate pathogen is highly host specific, although limited levels of infection among a few species of sympatric lepidopteran larvae are occasionally possible.

Use of *E. maimaiga* for Control

The major uses of *E. maimaiga* for control have been introductions of small amounts of field-collected resting spores to areas along the edge of spread of

gypsy moth. All indications suggest that the fungus will spread into these areas eventually but at present there are no data documenting how long this would take. When gypsy moth first colonizes a new area, population dynamics are chaotic along the leading edge of spread, and during this time land managers and the public are eagerly searching for means for controlling this pest. Cadaver- or soil-borne resting spores have been field collected and released in at least ten states.² In addition, cadavers bearing resting spores were collected in North America and released in Bulgaria, Siberia and possibly Romania (Hajek *et al.*, 2005).

Results from limited studies suggest that this fungus could be useful when applied in areas where it already exists to augment pre-existing levels of fungal inoculum and cause earlier initiation of fungal epizootics. However, to use *E. maimaiga* in this way, there would need to be a source of fungal inoculum. *E. maimaiga* is an obligate pathogen and is not easy to grow in the laboratory. Mass production of *E. maimaiga* is not feasible at present, although there are yearly requests for material to release in areas recently colonized by gypsy moth. The best stage for distribution is the resting spore, which is constitutively dormant after maturation. Although progress toward mass production of resting spores has been slow, these spores can now be produced *in vitro*; dormancy can be prevented and methods for storage have been developed. Time will tell whether methods for mass production of this fungus will be developed in the future to enable vastly improved availability.

Notes

¹ Classical biological control shipments today are maintained in quarantine before field release, in part to make sure that only the species of interest is released.

² Permits must be obtained from state and federal agencies before soil can be moved.

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8

Weevils Control Invasive Thistles in Canada

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Overview: Three species of the Eurasian biennial *Carduus* thistles have established in Canada as noxious invasive species. This is the story of the classical control of these weeds through introduction of their natural enemies from their original homeland.

The Weeds and the Problems They Caused

Three species of the Eurasian biennial *Carduus* thistles have established in Canada. One is not common, but, pre-biocontrol, nodding thistle (*Carduus nutans*) and plumeless thistle (*Carduus acanthoides*) formed pure, self-perpetuating stands on uncultivated grassland (Fig. 8.1). Nodding thistle, recorded in 1941 in Saskatchewan, had spread by 1967 to grassland in a 30,000 km² area (Harris and Zwölfer, 1971). Some stands were impenetrable, with 150,000, 2 m-tall plants/ha producing 150,000 seed/m². The slightly shorter plumeless thistle was a similar problem, mostly on limestone and gravel soils in Ontario and Québec. The thistles, the biocontrol agents and their biology are described on the website (Harris, 2000). The home page has links to other aspects of biocontrol discussed.

The Rationale for Classical Biological Control Agents for Thistle Control

The pioneer-climax model of plant succession indicates that the thistles, as pioneer species, will decline if grazing or disturbance is reduced. This is not true for many weeds introduced without natural enemies. These fit the state and transition model, with the weed 'state' returning after herbicide or mechanical control. Biological control is often a good solution for such introduced weedy species, and in Europe, with natural enemies, the thistles form temporary stands. Thus, these weeds were promising Canadian targets for classical biological control by establishing their enemies, as long as these enemies were restricted to the thistles.



Fig. 8.1. High density of nodding thistle in a Canadian pasture prior to the introduction of biological control agents.

A Promising Agent for Nodding Thistle Is Found, Studied and Introduced

Agriculture and Agri-Food Canada (AAFC) in 1962 contracted CABI to survey all European thistle species to obtain field host range information on the insects on both the target and other introduced thistles (Zwölfer, 1965). The most promising for nodding thistle was the seed-head weevil, *Rhinocyllus conicus* (Curculionidae: Coleoptera) (Fig. 8.2). Although competitively inferior to three other insects, high fecundity and wide egg distribution (dispersion coefficient 0.7) result in attack of up to 98% of the heads. The next best was the seed-head gall fly, *Urophora solstitialis* (Tephritidae: Diptera), which attacks up to 41% with a clumped distribution (dispersion coefficient 9.5) (Zwölfer, 1973).

CABI was then contracted to conduct host-specificity tests on a population of *R. conicus* at Mulhouse in the French Rhine valley. Single plant confinement showed the weevil nibbled on many plants; but normal feeding and egg development was confined to three closely related genera: *Carduus*, *Cirsium* and *Silybum*.



Fig. 8.2. *Rhinocyllus conicus* adult.

The oviposition range was similar, although eggs were laid on dummy heads of cotton attached to *Carduus* leaves. Larvae from transferred eggs did not penetrate into *Silybum* heads although it is a field host (Zwölfer and Harris, 1984). Field studies found the weevil consisted of populations with host preferences that were overridden by confinement or high weevil density (Zwölfer and Preiss, 1983). The Canadian and US review committees approved release in time for the 1968 season. Subsequently, the insect was released throughout North America, as well as in other countries.

In Canada, both nodding thistle flowering and weevil oviposition are synchronized to a short early summer period. The result was seed reduction of about 50%. Thistle stand perpetuation depended on the germinating seeds covering the ground with rosettes before grass encroached. Rosette densities of around 100/m² then self-thinned to 20/m² flowering plants. With less seed, grass gradually returned, preventing seedling establishment. Two Saskatchewan sites with 92/m² and 179 seed/m² in 1969 declined to 1.5/m² in 1981 and 2.5 seed/m² in 1982. The thistle now survives in pasture breaks, such as ground squirrel diggings. It is now rarely an agricultural problem but is still a nuisance in disturbed urban sites. The weevil is poorly synchronized with plumeless thistle, which has a later and longer flowering season; seed declined by only 15% and stands did not collapse. Similarly, nodding thistle was not controlled by introduction of the weevil in New Zealand and New South Wales, Australia, which have a long flowering season.

Releases of a Weevil against Plumeless Thistles Not a Success in Canada

A rosette weevil investigated as *Trichosiocalus horridus* (Curculionidae: Coleoptera) by Virginia Polytechnic Institute and State University was released against plumeless thistles in 1975 in Virginia with stock from Italy and in Canada with stock from Germany. This practice was a bad one, as recent studies found *T. horridus* to be a complex of three species. Fortunately, those from *Carduus* all seem to be *Trichosiocalus mortadello* (D. Bries, 2001, personal communication). It has been ineffective in eastern Canada, where oviposition is restricted to about 3 weeks in early spring, but a small plumeless thistle infestation collapsed in British Columbia. The weevil has two generations in Virginia, and plumeless thistle in mixed stands collapsed in 7–13 years, about twice the time taken to control the nodding thistle (Kok and Mays, 1991). Stock from Canada sent to New Zealand, and subsequently Australia, controlled nodding thistle with a 72% seed reduction. This increased to 81% where *R. conicus* and a seed-head fly, *Urophora solstitialis*, are also present (Woodburn, 1997).

Another Agent Found, Studied and Released against Plumeless Thistle

AAFC contracted CABI to test the gall fly *U. solstitialis*, as in Germany it attacks 60–75% of plumeless thistle heads. Stock from Austrian nodding thistle was released in 1991. In a mixed thistle stand in Ontario, both the fly and the weevil preferred nodding thistle. Attack of plumeless thistle heads in the autumn was 5% by *U. solstitialis* and 6% by *R. conicus*. By 1998, both nodding thistle and *R. conicus* had disappeared and *U. solstitialis* increased to attack 58% of the heads. The impact on seed production should be greater than an equivalent attack by *R. conicus*, as the gall is a powerful metabolic sink, which sequesters resources from all parts of the plant.

A Rust is Also Introduced

The USDA screened a rust disease of nodding thistle, *Puccinia cardorum* (Uredinales: Fungus), and obtained permission in 1997 for a limited release in Virginia. Limited releases are a fiction as by 1998 it had spread to California and in 1999 to Nevada. Releasing it in Canada would offer little gain, but it will arrive anyway.

Lessons Learned

The normal end of a biocontrol project is that a single agent species controls the weed in a habitat, in this case represented by different climates. However,

R. conicus has attacked native *Cirsium* spp. (Strong, 1977) beyond the expectation indicated by the tests of the Mulhouse population. Mulhouse stock did not establish on *Cirsium pycnocephalus* or *Silybum marianum* in California, but Italian collections from these thistles (Goeden and Ricker, 1977, 1985), as well as nodding thistle, have been established in the USA. Subsequent European allozyme studies showed that *R. conicus* separates into two groups with a genetic distance of 0.073 (Klein and Seitz, 1994). This is in the invertebrate subspecies range with weevils from *S. marianum* being in the taxon formerly known as *Rhinocyllus oblongatus*. There were also geographical differences within the two groups. Thus, genetically diverse stock has been established, but tests done only on those from Mulhouse.

Mulhouse stock in Saskatchewan is uncommon on *Cirsium* spp. unless with nodding thistle. It was not released in Alberta, where the wish was eradication with herbicides. The weevil in S. Alberta, apparently immigrated from Montana, which received both Mulhouse and central Italian stock, is presently common on the native *Cirsium undulatum* as well as the introduced Canada thistle. Both thistles remain abundant, and coincidentally with the weevil arrival, the rust disease, *Puccinia punctiformis*, became common on Canada thistle. Regulators no longer provide permits for the release of untested populations, and testing of new agents for thistle biocontrol in North America has ceased as researchers perceive that they will be rejected regardless of specificity.

The Ecological Risks and Benefits

Ecologically, the project has been beneficial, as range sites with pure thistle have returned to a species complex. A similar change following biocontrol has been documented for leafy spurge (*Euphorbia esula*) (Mico and Shay, 2002). Biocontrol reduced spurge canopy cover from 59% to 6%, increased plant diversity by 16 vascular plants, six of them natives, with native diversity increasing after 6 years. Spurge was displacing the northern prairie skink, the western spiderwort in its restricted Manitoba enclaves and the western prairie fringe orchid on the Sheyenne National Grasslands of North Dakota. Thus, both doing and not doing biocontrol may impact rare species. A US legal requirement that threatened or endangered natives should not be harmed is a sword over the reviewers' heads, and their safest course is to deny release even though the weed itself is a greater ecological threat. Preferably, reviewers will weigh the risks and select the least ecologically damaging course, as required in Australia. Plant acceptance by an agent is not always detrimental, as in spite of attack by the beetle *Aphthona nigriscutis* (Chrysomelidae: Coleoptera), the scattered native spurge, *E. robusta*, has increased following leafy spurge decline.

The native thistle receiving most concern is *Cirsium canescens*, which has a flowering phenology similar to that of nodding thistle (Louda, 1988). It is widespread in the central Great Plains, where it forms dense stands in disturbed areas, despite a 76–91% destruction of seeds by native insects and pathogens and death of 75–87% of the seedlings, partly from cattle grazing and trampling (Lamp and McCarty, 1981). Most of *R. conicus* impact must be at the expense of

the existing agents and is not added destruction. I am more concerned for *Cirsium pitcheri*, which is a threatened species confined to shoreline dunes along the western Great Lakes. It has the same enzyme loci as *C. canescens*, but a depauperate subset of alleles (Loveless and Hamrick, 1988). Possibly the greatest danger to *C. pitcheri* is hybridization with *C. canescens*.

Prediction of biocontrol risk relies on host range tests. North America still uses the no-choice test to demonstrate that the insect larva cannot mature on desirable species. The test worked when concern was limited to crop plants, but present concern for native relatives of the weed mean that 85% of the time the test results are broader than the field host range. Many of the remaining 15% may be species complexes, as in *T. horridus*, and need DNA analysis. The poor predictability arises because the tests are done on larvae. The most mobile insect stage is usually the adult, so it is up to the female to optimize her progeny survival by choosing appropriate plants. This trait is firmly held, with selection rewarding those getting it right. Externally feeding larvae can distinguish the host from interlaced vegetation, but not necessarily from close host relatives. New Zealand and Australia have changed their tests, but it is difficult in North America with two countries and many agencies whose representatives often have little insect ecology (see 'no-choice tests' and 'host specificity' on the website). Preferably *Carduus* biocontrol would have started with the release of *U. solstitialis*, but funds limit testing of several agents to select the most host specific.

This example shows that weed biocontrol with insects can have considerable ecological as well as economic benefits. Ecologically, there is no free lunch, as there will be changes, even if only those arising from replacement of the weed. Weed biocontrol is done by government in the public interest, which is more easily determined in economic than ecological terms. Indeed, it is not clear how to balance an endangered species threatened by the weed versus another by a biocontrol agent. Species of insects attacking weeds are not well known and may well be a species complex. Thus, it is essential to only release a tested population. Testing must involve the adult insect, which is usually responsible for field host selection. These are some of the technical problems, but there is also a political element, with different people having different interests and different fears.

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9

How Many and What Kind of Agents for the Biological Control of Weeds: a Case Study with Diffuse Knapweed

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Overview: Following its introduction to North America, diffuse knapweed came to occupy millions of hectares of rangeland. This is the story of biological control efforts done over 25 years before an effective agent was introduced. The moral is that for optimal success of biological control, thorough work should be done to understand the ecology of the target weed and its natural enemies. Effective agents are unlikely to be abundant in the native habitat, but are likely to be able to kill the plants in the exotic habitat.

Introduction

Classical biological control of weeds is the introduction of natural enemies from native areas to exotic sites where their host plants have become invasive and detrimental (overview in Myers and Bazely, 2003). Three important questions that confront biological control practitioners are: (i) which species of agent are safe to introduce; (ii) what types of agents are the most likely to be effective controls; and (iii) how many different species of agents should be introduced? Here I describe the biological control programme against diffuse knapweed, *Centaurea diffusa*, which involved the introduction of 13 species and the establishment of 11 agents over a 20-year period (Bourchier *et al.*, 2002). This is a story of a programme based on the premise that multiple species of agents would be necessary for successful control (Harris, 1981). After 30 years of monitoring diffuse knapweed, my students and I have observed that populations have declined following the establishment of the last agent to be introduced, the weevil, *Larinus minutus* (Fig. 9.1). The effectiveness of this species is demonstrated by the decline of diffuse knapweed density at sites with the beetle and continued high density of knapweed at sites where the beetle has not yet established. The diffuse knapweed programme provides an excellent case study for the evaluation of whether multiple species of biological control agent are required for success, or if single agent

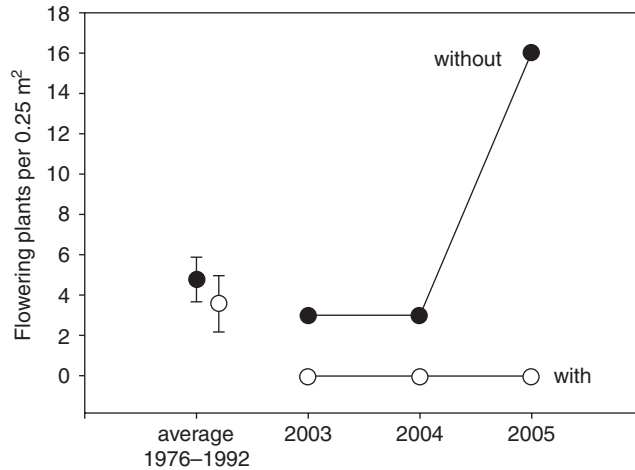


Fig. 9.1. Average number of flowering diffuse knapweed plants at a site where the weevil *L. minutus* was not established (solid circles) and a site where beetles became established in the late 1990s (open circles). Densities in recent years are compared to average densities measured between 1976 and 1992, prior to beetle establishment. Error bars are 2 SE. The return of average rainfall following several years of dry conditions was associated with the increased plant density in 2005 at the site lacking beetles.

species can be successful (Denoth *et al.*, 2002). It also provides the opportunity to study interactions among different agents and shows the value of quantitative monitoring of population densities for the evaluation of biological control success.

The Diffuse Knapweed System

Diffuse knapweed (*C. diffusa*) is an aster of Eurasian origin that was introduced to British Columbia prior to 1930 in contaminated lucerne seeds. Diffuse knapweed is a serious rangeland pest because it displaces forage grasses and has attained very high densities in dry rangelands in many areas of western North America. Knapweeds are poor forage for cows because of their bitter taste, and their dense stands are unpleasant even to walk through. Story *et al.* (2000) reported that in North America over 3 million ha of rangelands have been invaded by diffuse and spotted knapweed, *Centaurea stoebe* ssp. *microanthos* (formerly *Centaurea maculosa*).

The biological control programme for diffuse knapweed began in 1970 in Canada and, over the next 20 years, 12 insect species that attack different parts of knapweed plants (Fig. 9.2) were introduced, and nine of these are now widely distributed in British Columbia. The first two species to be introduced were gall-forming flies in the family Tephritidae, *Urophora affinis* and *Urophora quadrifasciata* (Harris and Myers, 1984), which have markedly reduced the production of seeds

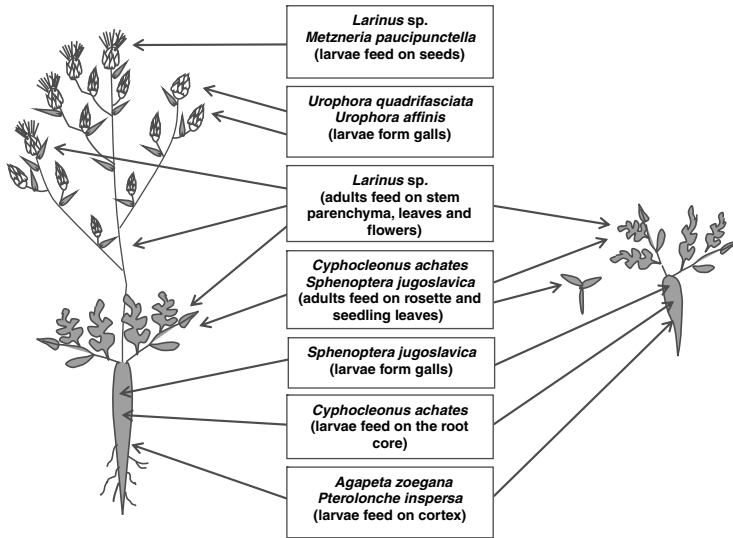


Fig. 9.2. Nine species of biological control agents attack various parts of knapweed plants. Both adult beetles and larvae of *C. achates*, *S. jugoslavica* and *L. minutus* feed on knapweed plants, while for other species only larvae attack the plants. Arrows indicate plant parts where agents oviposit and feed.

but not the density of knapweed plants (Myers and Risley, 2000, Myers and Bazely, 2003). *Metzneria paucipunctella*, a moth originally from European spotted knapweed, was also introduced in the early 1970s, and the larvae feed on seeds of both knapweed species but little is known of its impact (Bourchier *et al.*, 2002).

Next the root-boring beetle, *Sphenoptera jugoslavica*, was introduced and widely distributed in the 1980s, and these further reduced knapweed seed production (Powell and Myers, 1988; Powell, 1990), as well as the survival of seedlings and rosettes, and delayed the flowering of plants. A model based on density-dependent birth and death rates of the plants and including the impact of the beetles, however, showed that the knapweed populations were resilient to this attack (Powell, 1990).

In the 1980s to mid-1990s, three species of root-feeding Lepidoptera were introduced to and distributed in British Columbia. *Agapeta zoegana* was first introduced in 1982, distributed through the 1990s and has become widely established (Bourchier *et al.*, 2002). *Pelochrista medullana*, introduced in 1992, has not thrived (Bourchier *et al.*, 2002). *Pterolonche inspersa* apparently became established following its initial introduction in 1986, although it has not been monitored (P. Harris, Lethbridge, Alberta, 2006, personal communication). Although initially collected from spotted knapweed, the weevil, *Cyphocleonus achates*, was widely distributed following its original introduction to British Columbia in 1987. It can exist on large rosettes of diffuse knapweed and now occurs at many sites in the southern Okanagan Valley of British Columbia, even though adult beetles are not capable of flight. Larvae of this weevil feed on the root core

and reduce the growth of flowering plants and can kill rosette plants (Story *et al.*, 1996; Bourchier *et al.*, 2002).

Most recently, beginning in 1991, two species of weevil, *Larinus minutus* and *L. obtusus*, were introduced as biological control agents on diffuse and spotted knapweed (Groppe, 1990) (Fig. 9.3). The adult weevils damage and can kill plants by feeding on the epidermis of stems and branches of the knapweed as well as feeding on flower buds, thereby causing their abortion. The larvae feed on developing achenes. The *Larinus* species were widely distributed in British Columbia in 2000 and 2001 and they appear to be good dispersers.

Two additional species that were introduced, but did not establish, are the ovary-feeding fly, *Chaetorellia acrolophi*, and the soft achene-feeding fly, *Terellia virens*. A fungus, *Puccinia jaceae diffusae*, was accidentally introduced to British Columbia, and this can kill seedlings and rosettes as well as infect flowering plants. Although it is widely spread it is not known to have an impact on plant density (P. Harris, Lethbridge, Alberta, 2006, personal communication).

The same species of potential biological control agents were introduced to knapweed infestations in the USA and a recent overview is given by LeJeune *et al.* (2005).

Interactions among Agents

One reason for being conservative in the introduction of biological control agents is that a potentially effective agent may be suppressed through competition by another and thus reduce the success of the biological control programme. In fact, Zwölfer (1973) recommended the introduction of poor competitors for biological weed control, under the assumption that once released from competition, they would thrive and damage host plants. I expanded this idea and suggested that agents that are rare in their native habitats may have the best potential for biological control success. My students and I reviewed weed biological control programmes to determine if single species of agents could be successful and if competition among species of agents was indicated. We found that the establishment rates of agents were not significantly related to the number of agents introduced, although for single-agent introductions, the success of establishment was 53% and for multiple-species programmes it was 32% (Denoth



Fig. 9.3. *Larinus minutus* on a knapweed plant.
Photo by Shannon White.

et al., 2002). Thus there is no strong evidence that the introduction of multiple species influences the establishment of agents.

We also found that the success rate of biological weed control increased with the number of agents introduced (Denoth *et al.*, 2002). We suggested that this result could be explained by either the 'lottery model', in which the probability that a successful agent will be introduced increases with the number of agent species introduced, or the 'cumulative stress model' (Harris, 1981), in which additive stress from multiple agents promotes host plant decline. Perhaps our most important finding was that in over half of successful programmes, success was attributed to a single agent. This supports the proposal that cumulative stress from multiple agents is not necessary for successful biological control, but in many cases 'silver bullets' can achieve success.

The multitude of species introduced on diffuse knapweed allows the study of interactions among agents. The agents introduced on knapweed fall into two categories: those that reduce seed production directly by attacking the flowers and those that reduce seed production indirectly by feeding on the roots of the plants and thus reducing their growth, development and energy stores for seed production (Fig. 9.2).

Seed-feeding Species

Given the high number of agent species introduced, it is interesting to determine if competitive interactions can occur among agent species in the diffuse knapweed programme. The two gall flies attack the knapweed flowers at slightly different times. *U. affinis* lays eggs in flower buds and is reported to be competitively superior to *U. quadrifasciata*, which lays eggs at a slightly later stage of bud development. The two species also differ in that *U. quadrifasciata* has a larger second generation than *U. affinis*, which is primarily univoltine. This might contribute to increased dispersal of the latter, as they must seek out flower buds at the proper stage for oviposition. *U. affinis* also produces a hard gall, which may protect it from predators, while *U. quadrifasciata* produces a potentially more vulnerable soft gall. In territorial interactions between males of the two species, *U. affinis* is dominant (Berube, 1980). *U. quadrifasciata* and *U. affinis* were initially introduced together, with *U. quadrifasciata* being a contaminant of the *U. affinis* stock. However, both species increased rapidly (Harris and Myers, 1984) and they coexist in most locations (Harris, 1990).

Larvae of the moth *M. paucipunctella* will feed on *Urophora* larvae (Story *et al.*, 1991); however, given their poor survival, *M. paucipunctella* is not a major player in the species interactions. *L. minutus* larvae kill *M. paucipunctella* larvae in co-inhabited flower heads (Harris, 2005; website). These negative interactions among species in the seed-feeding guild may increase the dispersal of agents as they search for suitable hosts and stages of bud and flower development for oviposition. They have not, however, apparently prevented the establishment of species.

Finally, two species of *Larinus* were introduced to British Columbia, *L. minutus* and *L. obtusus*, and nothing is known about their competitive interactions. Harris (Lethbridge, Alberta, 2006, personal communication) has suggested that the two

species may hybridize. The original introductions were based on differences in host plant and habitat preferences, *L. minutus* preferring diffuse knapweed and drier environments, and *L. obtusus* preferring spotted knapweed and moister environments. Understanding the interactions between the two species will require genetic studies.

U. affinis may also have an advantage over the seed-feeding weevil *L. minutus* because buds heavily attacked by *U. affinis* do not open and are not attractive to ovipositing weevils (Harris, 1990). Feeding on the stems, leaves and buds of knapweed plants by *L. minutus* in the early spring, however, can reduce bud development and thus oviposition sites for flies. On the other hand, *L. minutus* larvae will feed on gall-fly larvae if they are in the same flower head (LeJeune *et al.*, 2005). LeJeune *et al.* (2005) found 'no detectable effect of *U. affinis* on seed abundance' of knapweed, but *U. affinis* was reduced by the presence of *L. minutus*.

Root-feeding Species

Associations among insects feeding on knapweed roots were studied in field surveys in Europe, their native habitat, by Muller (1989). He found 12 species of insects attacking five areas of the roots of diffuse knapweed: crown, collar, vascular tissue, cortex and outer surface. Of these, the most relevant to the knapweed biological control programme are the two widely established species that attack the central vascular tissue of the root, the weevil, *C. achates* and the buprestid beetle, *S. jugoslavica*. Larvae of the moth, *A. zoegana*, develop in the root cortex. No evidence for negative interactions among these species was apparent in Muller's study, and *C. achates* and *A. zoegana* were positively associated in roots, an indication that they may have similar preferences for larger plants as oviposition sites. LeJeune *et al.* (2005) found that *C. achates* responded positively to larger knapweed rosettes that resulted from nitrogen fertilization, but that *S. jugoslavica* avoided fertilized plants. *A. zoegana* was not part of this study.

Most of the work on the root-feeding species in North America has been on spotted knapweed. In British Columbia, the most common of the species that attacks diffuse knapweed roots is the buprestid beetle *S. jugoslavica*. This is also the most widely distributed of the root-feeding species in British Columbia, and at one site they attacked half of the knapweed plants (personal observation). This is considerably higher than the 2% level of attack reported by Muller (1989) for one European site. The lower density and more restricted distributions of the other root-feeding species reduce the opportunity for competitive interactions among these species in British Columbia at this time.

What Types of Agents Should be Introduced?

Early in this study, Harris (1974) wrote a paper describing a system for the evaluation of different types of insects as biological control agents. This was based on the type of damage they did. An alternative approach to selecting biological control agents is from the perspective of the plant biology.

Attack by the gall flies and the root-boring beetle *S. jugoslavica* reduced the seed production of diffuse knapweed by over 95% without reducing plant density (Powell, 1990). This may seem quite surprising, but models of the density-survival patterns of diffuse knapweed showed that compensatory survival and seed production buffers population densities from increased seed predation (review in Myers and Bazely, 2003). Survival is reduced when density is high and better when density is low, so populations can be maintained at high densities even with reduced numbers of seeds. In addition, at low density, flowering plants are larger and produce more seeds, and this too helps to buffer population densities. To explore these associations we developed a simulation model that incorporated the impacts of the gall flies, the root-boring beetle *S. jugoslavica*, and a hypothetical agent that killed plants after the stage at which compensatory survival would occur, e.g. the rosette or flowering-plant stage (Myers and Risley, 2000). This model showed that only the latter mortality would reduce knapweed density, and we recommended the introduction of species that could kill plants. Two of the agents that are established in British Columbia can kill plants: *C. achates* and *L. minutus*, and thus these fit the predicted requirement for successful control of diffuse knapweed.

L. minutus was the last of the species to be distributed and established on diffuse knapweed. In many sites it has reached high population densities. Particularly during a period of drought, feeding by beetles killed many bolting plants. This result has also been observed in other areas (LeJeune *et al.*, 2005). With this attack, densities of diffuse knapweed have declined precipitously at many locations in British Columbia. While it appears that *L. minutus* caused the decline, the widespread presence of the *Urophora* species and of *S. jugoslavica* make it impossible to evaluate if the weevil *L. minutus* could have achieved biological control success on its own and experiments are necessary.

Conclusions

Over the 30 years of the biological control programme on diffuse knapweed, interest in non-target impacts of introduced biological control agents has increased (Louda *et al.*, 2003). In the diffuse knapweed programme, 13 additional exotic species have been introduced in the quest to reduce the density of one non-indigenous host plant. The introduction of multiple agents in biological control programmes raises an interesting philosophical question about the potential modification of species complexes. With each introduction comes a chance of indirect effects on non-target plants or influences on other components of the community, such as by providing new food items for predators.

We anticipated in the early stages of the knapweed programme that seed predators would be unlikely to control diffuse knapweed effectively. And yet *L. minutus* was introduced approximately 20 years after the gall flies, primarily as an additional seed predator. It seems that the main impact of *L. minutus* may be the severe damage caused by adult weevils feeding on flowering plants. This impact received little comment in the studies carried out in Europe prior to the introduction of *L. minutus* to North America (Groppe, 1990). Perhaps further

study of this species in its native habitat would have helped to identify its multiple positive characteristics, such as adult feeding, high reproductive potential and good dispersal ability, in addition to its impact as a seed predator.

Although competitive interactions among the multiple biological control agents in the diffuse knapweed programme display occur, these have not apparently caused species exclusion. Variation in the density and phenology of host-plant populations may allow the established species to coexist. Of the root-dwelling species only *S. jugoslavica* is currently widely distributed at moderately high densities. If densities of *C. achates* and *A. agoena* increase, more competitive interactions may occur.

Earlier I posed three questions that are important to biological control and these can be considered retrospectively for the knapweed programme.

- 1.** Were the introduced species safe? No detrimental non-target impacts of the biological control agents in the knapweed programme have been recorded. Thus the safety of the agents appears to have been evaluated appropriately.
- 2.** What types of agents were most likely to be successful? Because knapweed produces many seeds it was thought initially that seed predators or insects that reduced seed production would be effective. However, the ability of knapweed to compensate made populations resilient to seed predation. Although we predicted that only an agent that killed plants in late life-history stages could be successful, this type of agent was not initially identified. Early selection of *L. minutus* may have reduced the number of agents introduced.
- 3.** How many agents should be introduced? It is not clear that this question was considered in the knapweed project. The economics of identifying and testing agents for host specificity is always a consideration. In the knapweed programme, however, the philosophy was to keep introducing agents until success was achieved. While this approach has been common to most programmes in biological control of weeds, recent concerns arising from the attack of native plants by introduced insects may change this philosophy in the future. If a limit on the number of introductions were set, more focus might have to be placed on finding effective agents at the pre-release stage.

Like most biological control projects, this programme has involved concerted efforts by many people in Canada and Europe. It is an example, however, of how difficult it is to maintain long-term monitoring, which is crucial to its evaluation. Measuring only seed production is an insufficient index of biological control success, which is only achieved through reduced plant density. It is unfortunate that other exotic species now await their opportunity to replace knapweed. In particular, the grass *Bromus tectorum* appears to have benefited by the knapweed decline.

Although in almost half of the successful biological control of weeds programmes, success has been attributed to a single species of agent, the general support for the possibility that 'silver bullets' exist remains weak among biological control practitioners. I think that a more conservative and predictive approach is necessary for selecting agents in the future programmes, to reduce the number of exotic species introduced. In addition, my suggestion that seed predators are unlikely to successfully control weeds that produce many seeds has not been popular. Field experiments and population-simulation models of target

weeds in both exotic and native habitats should be prerequisites for selecting the agents for introduction. And finally, I recommend that more emphasis should be placed on species that are rare in their native environment rather than those that are common and widely spread when picking candidate species for biological control. It is likely that plants are well adapted to the attack of their common insect herbivores. This too has not been a widely accepted suggestion. A more scientific and experimental approach to biological weed control could reduce the economic and environmental costs of introducing unsuccessful agents in future control programmes.

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10

Why is Biocontrol of Common Ragweed, the Most Allergenic Weed in Eastern Europe, Still Only a Hope?

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Overview: This chapter presents the story of a long and as yet unsuccessful struggle to find suitable fungal and/or insect biocontrol agents for ragweed, a plant that became a widespread allergenic weed in Eastern Europe. This effort illustrates why biocontrol initiatives, although desirable, sometimes cannot be implemented into practical control strategies, in spite of the fact that they might represent a realistic solution to problems affecting human health, the environment and agricultural productivity.

Introduction

Ambrosia artemisiifolia (Fig. 10.1), or common ragweed, has in the last decade become the best-recognized weed species in Eastern Europe. This arose because so many people developed allergies to the air-borne pollen of this weed that the governments had to initiate programmes to bring attention to this noxious weed. They did this through a series of advertisement campaigns utilizing huge posters, brochures, magazine articles, school programmes and other methods (Figs 10.2 and 10.3). Current estimates suggest that between 10 and 15% of the population are now allergic to ragweed where it has established in Eastern Europe. In North America, where this weed originates from, ragweed allergies are well recognized (Bassett and Crompton, 1975) but until the 1990s this illness was relatively rare in Europe. In the affected Eastern European countries, the health costs associated with ragweed allergies are paid by the national health insurance systems and the costs of control in urban areas by the local councils. These costs were of such magnitude that authorities were forced to act at both the regional and national levels to deal with the problem. For example, the Hungarian Parliament identified ragweed as a national health threat and made control of ragweed a law. Furthermore, in 2003 the Hungarian taxpayers were given the opportunity to direct 1% of their annual tax for ragweed control. Unfortunately, none of these actions have resulted in a decrease of ragweed pollen or ragweed populations, as these continue to spread along with the number of people developing allergies.



Fig. 10.1. A ragweed plant in the front of Pont du Gard, a famous Roman aqueduct belonging to the UNESCO World Heritage. Ragweed has only recently colonized this area, which is a popular natural reserve in southern France.

Spread of Common Ragweed in Europe: an Example for Biological Invasion Caused by an Alien Weed Introduced to a New Environment

The seeds of *A. artemisiifolia* were introduced in large numbers into Europe, starting from the 19th century as contaminants of agricultural products, such as cereals, imported from the USA and Canada, where ragweed is native. Ragweed plants are poor competitors compared to many other weed species and they only produce seeds late in the season. They probably did not establish well in most parts of Europe but some must have survived in a few places, from where they slowly spread without causing much trouble or notice. Until the 1970s, ragweed was just one among many weed species present in agricultural fields in some parts of Europe. Mechanical and herbicide treatments controlled its populations in cultivated fields and it created no noticeable issues outside of agricultural areas. This is not surprising as ragweed is a primary colonizer of disturbed and abandoned fields (Bassett and Crompton, 1975), and such sites were not common in either Western or Eastern Europe until the end of the 20th century.

A boom in its spread in Eastern and Western Europe, which started approximately 15 years ago, was facilitated by socio-economic factors. In Eastern Europe,



Fig. 10.2. 'National ragweed control project for everyone's health' says a huge poster exhibited by the National Ragweed Control Committee in the middle of a weedy area in Hungary. *Artemisia vulgaris*, another highly allergenic weed, is growing in the front of the poster. The efficacy of this kind of propaganda is extremely doubtful.

these could be linked with the deep political changes that led to the formation of young democracies in that region. During this process, many socialist-type agricultural cooperatives were closed and their lands were subdivided and re-distributed to their former owners or descendants, who, in many cases, did not continue to cultivate them for years. Thus, large, formerly well-kept agricultural fields became abandoned and were quickly colonized by ragweed. In addition, construction of new roads, motorways, shopping centres, etc. soon followed, but with little effort spent on landscape management. This also created large disturbed areas, where ragweed readily became established. In less than a decade, ragweed became the most widespread weed species within both agricultural and urban areas in Hungary, as well as in many neighbouring countries except Austria, where landscape management standards remained in place.

Parallel to the explosion in the spread of ragweed in Eastern Europe, its populations have been found to be rapidly expanding along the Rhône River in France, as well. This was thought to be an unwanted result of a policy of the European Community, established in 1994, which required French farmers to either stop growing crops in certain lands or grow sunflowers (Déchamp and Méon, 2002). Both requirements facilitated the spread of ragweed as its control is particularly difficult in sunflower fields.

The massive spread of ragweed in different parts of the world was always correlated with an increase in areas disturbed by humans. For example, analysis



Fig. 10.3. 'Ragweed-free street' announces a plate in Budapest in a street covered with asphalt.

of pollen in soils from Canada showed that in the 18th and 19th centuries the increased agricultural activity as result of settlement by the Europeans coincided with an increase in ragweed pollen in those regions (Bassett and Crompton, 1975).

Natural Enemies of Ragweed in Europe and North America

Biological invasions by alien plant species into newly occupied areas can sometimes be explained, at least in part, by their 'escape' from natural enemies (insect herbivores, fungal, bacterial and viral pathogens, etc.) that they would normally encounter in their native habitats (Evans *et al.*, 2001). Nothing, however, was known as to what natural enemies might exist in Europe that could control ragweed populations in the newly occupied areas. In 1994 my colleagues, Dr Gyula Bohár and Dr László Vajna, started to look for natural control agents of *A. artemisiifolia* in Hungary. I joined their project in 1997. Our studies showed that only a few plant pathogenic fungi infect ragweed in Hungary, some of which were identified on *Ambrosia* for the first time in Europe (e.g. Bohár and Kiss, 1999; Vajna *et al.*, 2000). In the USA, however, more than 25 fungal pathogens are listed as pathogens on *A. artemisiifolia* (Farr *et al.*, 1989). We found only a few polyphagous arthropods (insects that feed on many kinds of food) that fed on ragweed in Hungary or in other European countries (Balázs Kiss, unpublished data). More than 200 species, however, are known to damage *A. artemisiifolia* in North America, including a large number of polyphagous species and some oligo- and

monophagous arthropods, as well (Harris and Piper, 1970). Recently, a reciprocal transplant assay showed that experimental ragweed plants suffer much less herbivore damage in France than in Canada (Genton *et al.*, 2005).

Biocontrol of Ragweed in Europe: a Reasonable Approach to the Problem

All the data we and others accumulated suggested that the European ragweed population escaped from most of their natural enemies. Specialized fungal pathogens found in North America, such as rusts and powdery mildews, or arthropods that feed mostly or exclusively on ragweed were not present in Europe. Our field surveys conducted regularly since 1994 in Hungary confirmed that ragweed plants remained perfectly healthy in the field, except for outbreaks of epidemics caused by two fungal pathogens, *Phyllachora ambrosiae* in 1999 (Vajna *et al.*, 2000) and *Plasmopara halstedii*, (a downy mildew) in 2002 (Vajna, 2002). However, similar epidemics were not observed in other years and we couldn't find any other biotic factors causing damage to ragweed in Europe.

Noxious alien weeds have been successfully controlled in some newly invaded areas by artificial release of selected natural enemies, such as insects and plant pathogens, called biocontrol agents (BCAs) (e.g. Evans *et al.*, 2001). These are generally collected from the countries of origin of the invasive species. The release of BCAs requires the accumulation of data ensuring that the BCAs are, indeed, specialized to the target species and won't attack other plants (Seier, 2005).

Speculations about Potential BCAs of Ragweed in Europe

Based on information from the literature, our initial idea was to identify and to release a rust fungus, such as *Puccinia xanthii*, to reduce the spread of ragweed in Europe (Bohár, 1996). This fungus produces only teliospores (blackish, thick-walled spores of some rusts) and occurs on *Xanthium* spp. in North America, parts of Europe and Australia (Morin *et al.*, 1993). In addition, it is known to infect *A. artemisiifolia* and *Ambrosia trifida* in the USA (Batra, 1981; Farr *et al.*, 1989). However, neither *P. xanthii* nor any other rust species have been recorded on ragweed outside North America thus far. Although *P. xanthii* is present in Hungary on *Xanthium italicum* (Dávid *et al.*, 2003), our efforts to infect *A. artemisiifolia* with this rust either in the greenhouse or in the field have always failed (Dávid and Kiss, unpublished results). Thus, the release of an American strain of *P. xanthii* specific to *A. artemisiifolia* could be envisaged in Europe. Another form of *P. xanthii* has already been recommended as a classical BCA of *A. trifida* outside North America (Batra, 1981).

Some other fungal pathogens that could be considered as potential BCAs of ragweed in Europe include *Protomyces gravidus*, a North American fungus specialized to *A. artemisiifolia* and *A. trifida*. This pathogen sometimes causes serious epidemics on ragweed, and it was evaluated as a mycoherbicide against the

two *Ambrosia* species. However, the results obtained showed no control (Cartwright and Templeton, 1988) and this aspect of the project was discontinued. *Sclerotinia sclerotiorum* (Bohár and Kiss, 1999) or a *Phoma* sp. (Teshler *et al.*, 2002), two broad-spectrum fungal pathogens, were also considered as BCAs of ragweed, but their use would have to be limited to specific non-agricultural conditions.

The North American insect herbivores of ragweed have been studied for biocontrol purposes far more intensively than plant pathogens, and a number have been released as control agents in different parts of the world (Julien and Griffith, 1998). The most studied BCA of ragweed, a monophagous North American leaf beetle, *Zygogramma suturalis*, was released at locations in the former Soviet Union (Kovalev, 1989), Croatia (Igrc *et al.*, 1995), China (Wan *et al.*, 1995) and Australia (Julien and Griffith, 1998). They successfully established and spread in the former Soviet Union and the initial results were promising (e.g. Kovalev, 1989). However, their populations remained low and their overall impact was minimal (Reznik *et al.*, 1994). In areas of China 30,000 beetles were released from 1988 to 1991, but only a few individuals were recovered, indicating a poor establishment (Wan *et al.*, 1995). Similar results were obtained in Croatia (Igrc *et al.*, 1995). Thus, *Z. suturalis* did not fulfil expectations as a BCA against *A. artemisiifolia*.

A Search for Natural Enemies of Ragweed in North America and the Missing Rusts

To our knowledge, there have been no attempts to find BCAs for ragweed in North America thus far, except for Dr Kovalev's survey in the 1970s, which led to the release of a leaf-eating beetle, *Z. suturalis*, in the former Soviet Union in 1978 (Kovalev, 1989; Reznik *et al.*, 1994). In 2003, I received an OECD fellowship allowing me to spend 3 months in Canada and the USA to look for potential BCAs of *A. artemisiifolia*. I travelled over 13,000 km, from Québec to North Carolina and back along the eastern coast of North America, and from Québec to Wisconsin, searching for both plant pathogens and arthropods that could be considered as potential BCAs of ragweed for Europe. Much to my surprise, I did not find any ragweed plants infected with rusts, although this was a priority for my explorations. I was specifically looking for *P. xanthii*, a fungus reported to infect ragweed in the USA (Batra, 1981; Farr *et al.*, 1989). When I realized that this task could not be achieved, I contacted over 20 colleagues working at universities, USDA and extension services in Canada and the USA and asked them to look for rusts and other fungal diseases on ragweed. In addition, I published a similar request in *Inoculum*, the newsletter of the Mycological Society of America (vol. 54 (5), page 23, 2003). Though I received numerous replies and many searches were made, no rust fungi were found on ragweed by anyone.

To assure myself that *P. xanthii* does infect *A. artemisiifolia* in North America, as reported, I borrowed and examined all the herbarium materials of *A. artemisiifolia* infected with rust fungi from U.S. National Fungus Collections (abbreviated as BPI and located at USDA-ARS, Systematic Botany and Mycology Laboratory,

Beltsville, MD). Based on the morphology of the telia and teliospores found on the dried leaves, I identified 11 BPI specimens collected between 1855 and 1963 in five states of the USA (FL, KS, OK, SC and TX) as *A. artemisiifolia* plants infected by *P. xanthii* (Fig. 10.4). This supported the published information on the occurrence of *P. xanthii* on common ragweed in these places (Farr *et al.*, 1989). Unfortunately, the limits of my budget did not permit me to re-visit the places where these herbarium materials came from. However, rust fungi can easily spread for long distances, so we cannot explain why *P. xanthii* was missing from *A. artemisiifolia* in the searched regions of the USA and Canada, although it was found between 1855 and 1963 in Florida, Kansas, Oklahoma, South Carolina and Texas by those who have deposited herbarium specimens at BPI.

The Most Promising BCA: *Ophraella communa*, a Leaf Beetle

During my survey in Canada and USA, the most widespread natural enemy of *A. artemisiifolia* turned out to be a leaf-eating beetle, *Ophraella communa*. Its larvae, pupae and adults were present on ragweed everywhere I went. Sometimes the larvae caused serious damage to plants in the field. *Z. suturalis* was also present on ragweed but its population densities were much lower than those of *O. communa*. Although I am a plant pathologist, I became interested in *O. communa* and have started to learn more about it. This species, known from North America only, was recently discovered in Japan (Yamazaki *et al.*, 2000). It has been investigated as a potential BCA of ragweed in Australia, but Palmer and Goeden (1991) did not recommend its release there because it caused some damage to sunflower plants in a no-choice test. This report excluded *O. communa* from the list of potential BCAs for ragweed outside North America. However, their conclusions came from results of a single small-scale no-choice test and placing such exclusion on this insect may have been premature. Palmer and Goeden (1991) themselves noted that *O. communa* has never been recorded as an insect pest of sunflower in North America, where both *A. artemisiifolia* and sunflower are found in abundance. Recently, Dernovici *et al.* (2006) re-examined the feeding behaviour of *O. communa* on sunflower and *A. artemisiifolia* in more detail using choice and no-choice tests and found that ragweed is the main host plant of this beetle. This study showed that *O. communa* cannot complete its life cycle and cannot increase its population on sunflower plants, although under no-choice tests it can cause some damage to sunflower. In Japan, where *O. communa* was first found in 1996, detailed studies of the feeding behaviour of this beetle also showed that *A. artemisiifolia* is the preferred host plant (Yamazaki *et al.*, 2000) and that no damage occurs to sunflower.

O. communa has performed well as an inundative BCA of ragweed in cabbage and carrot fields in Canada (Teshler *et al.*, 2002). A special device was designed for its collection, storage and delivery (Teshler *et al.*, 2004), which was used in inundative biocontrol experiments. I used this device during my trip in 2003 to Canada and the USA to collect and transport many individuals, and

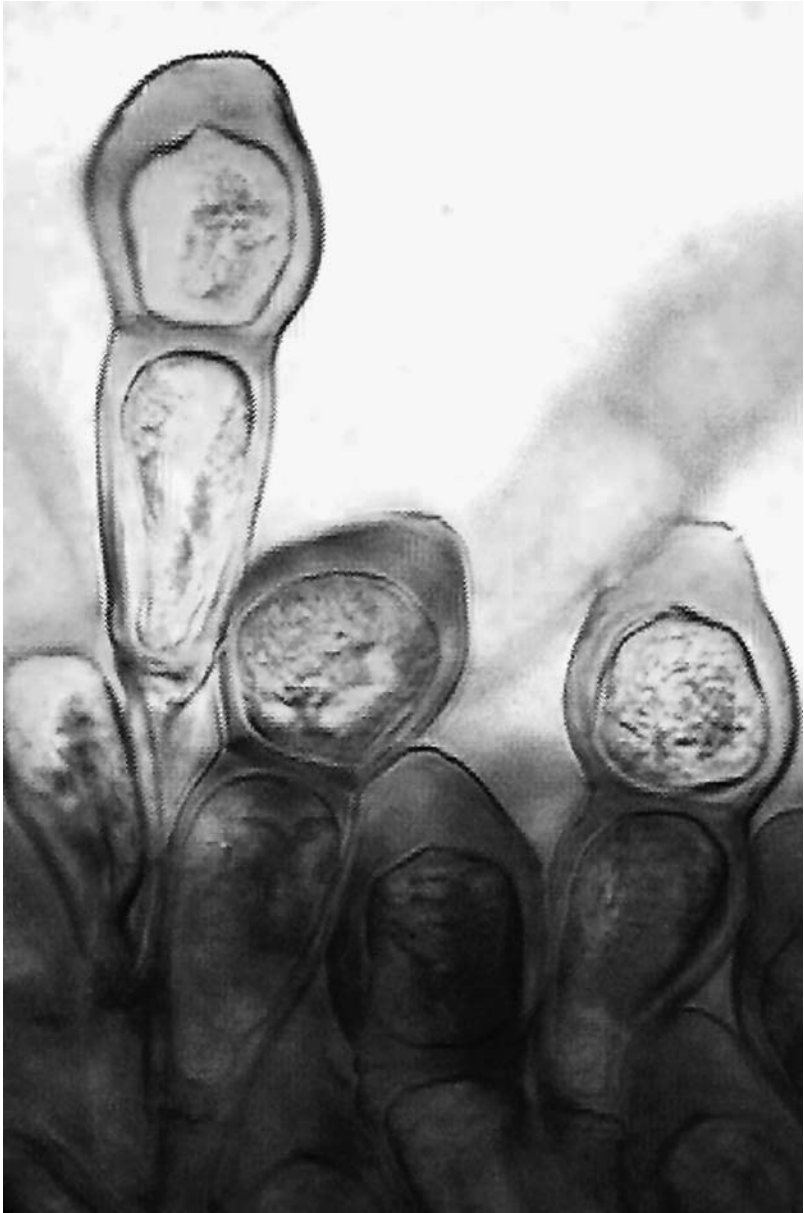


Fig. 10.4. Teliospores of *P. xanthii* from a herbarium specimen of *P. xanthii* infecting *A. artemisiifolia* collected in Texas in 1915. (Courtesy of Dr L. Vajna)

thanks to Dr Teshler's guidance, I learned a simple mass-rearing technique for *O. communis*. Within a few weeks I obtained hundreds of beetles at McGill University, which I planned to take back with me to Hungary to continue my studies in a quarantine laboratory. However, this did not happen, as the permit

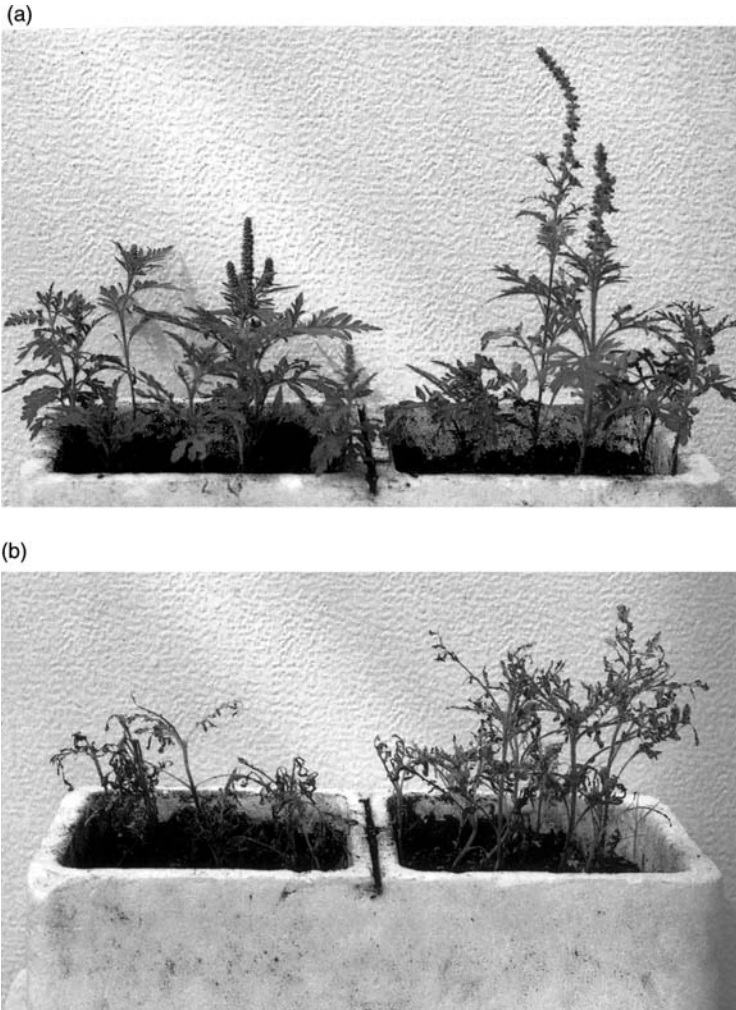


Fig. 10.5. Potted ragweed plants used to test the efficacy of *O. communis*, a leaf-eating North American beetle specialized to *A. artemisiifolia*, against this noxious weed. **a.** Healthy plants. **b.** The same plants defoliated by *O. communis* larvae in 2 weeks.

necessary for their import was not issued. At this stage, I think *O. communis* could be one of the most promising candidates for a BCA of ragweed for Europe (Fig. 10.5).

The Role of Media in Popularization of a Planned Biocontrol Project

Soon after our first proposals to implement a biocontrol project against ragweed were rejected in Hungary, my colleagues and I started to write articles in Hungarian

journals of popular science to spread the idea of the concepts of biocontrol of ragweed. Journalists became very interested in this initiative and we received extensive coverage on TV, radio, popular daily and weekly papers, women's magazines, etc. Titles of articles written by journalists (not by us!) included: 'Who will defeat ragweed?', 'Operation against weeds', 'Only natural enemies can help', and so on. Our proposals to adopt BCAs against ragweed are still widely cited by the Hungarian media. However, this did not help us to receive funding for this project.

Frustrating Experiences with Authorities and Funding Agencies

Based on the magnitude of the problems caused by ragweed in Eastern and Western Europe, one might expect that all available control methods would be considered by the decision makers in charge of this issue. Although our initiatives concerning ragweed biocontrol were well known, all our applications for national and European Union (EU) grants were rejected. Moreover, our applications to obtain permits to import *O. communa*, *Z. suturalis* and *Pseudomonas syringae* pv. *tagetis* from North America, under strict quarantine conditions, were also refused by the Hungarian authorities. This was ironic in the case of *Zygogramma* beetle, as it was released in neighbouring Croatia many years ago (Igrc *et al.*, 1995) and might have entered Hungary without a permit. The only import permit we were given was to import rust fungi under quarantine conditions, but rusts were not found in North America. The attitude towards our initiatives is probably due to a general fear of biological control methods of any kind, especially when it comes to introducing alien species (e.g. Seier, 2005). These concerns are sometimes valid but they can also mislead decision makers not to support any such proposals. Our efforts on ragweed biocontrol were supported by minor grants or by our own funds as this study has become a kind of hobby for us. Of course, no serious projects could be built up in this way.

In contrast to the USA, Australia, South Africa and New Zealand, where a number of successful classical biocontrol projects against introduced weeds have already shown the values of this method, Europe still lacks any experience with fungal BCAs of weeds (Seier, 2005). The first two projects on such topics have only recently started in the EU (Seier, 2005). This could also be a reason why authorities and funding agencies at national and European level haven't supported any initiatives to develop a biocontrol project against ragweed so far.

Concluding Remarks

At this stage we don't know whether it is possible to develop a biocontrol method to suppress ragweed populations in Europe. For many noxious weeds, no effective BCAs have yet been found, and *A. artemisiifolia* might be one of the species which simply cannot be controlled with BCAs. However, the list of potential insect and fungal BCAs of ragweed already identified suggests that it would be worth trying.

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11 Biocontrol for Everyman: Public Participation in a Weed Project

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Overview: Purple loosestrife is a European perennial plant that was introduced into the New World almost two centuries ago. It is found from coast to coast in North America and is considered a serious weed of wetlands. This is the story of getting the public involved in a team approach that includes schools, landowners and managers, who collect, rear and redistribute the introduced biological control agents in a classical biological control approach for this invasive weed.

Introduction

In the 15th-century English morality play *Summoning Everyman* the protagonist (Everyman) is on a quest but finds little help from his friends. In calling for help, Everyman says:

Commanded I am to go on a journey,
A long way, hard and dangerous,
And give a strait count without delay. . .

Forsaken by his friends, Everyman finds solace in his journey from his one true supporter, Good Deeds.

We are using the story of Everyman as an allegory, relating our work on a biological control project to the medieval tale. Then, as now, the term ‘everyman’ was used in reference to the common, everyday person.

Biological control projects, while seldom ‘hard and dangerous’, are often long and difficult journeys. Many projects take years from inception to success, and require diligence and support along the way, as well as the solace from ‘Good Deeds’ – our belief that we are doing the right thing. We relate here a project in which we have involved the public – our latter-day Everyman – by

engaging them to help us carry out numerous aspects of the project and to 'give a strait (sic) count without delay.'

Biological control needs to be conducted by specialists, especially the search for and importation of exotic natural enemies, to ensure safety and legal compliance with permit requirements. However, many biological control projects over the years have benefited from the inclusion of non-specialists to broaden participation and increase the likelihood of success. The project targeting leafy spurge (*Euphorbia esula*), for example, a weed of pastures and natural areas across the northern areas of the USA, has benefited from a team approach that includes land-owners and managers, who collect beetles for redistribution. Broadening participation gives the public a chance to learn about the rationale behind and the nuances of biological control projects, as well as providing participants with a sense of ownership of the project.

Purple Loosestrife, an Invasive Species in North America

Purple loosestrife (*Lythrum salicaria*) is a European perennial plant that has been in the New World for almost two centuries. Found from coast to coast in North America, especially in the northern half of the USA and southern half of Canada, it is considered a serious invasive weed of wetlands by many land managers. Large loosestrife plants have been estimated to produce over 2 million seeds each year, with the seeds remaining viable for years. With few herbivores to feed on it, purple loosestrife can grow unchecked, eventually forming dense stands in which little else will grow.

Although beautiful in flower, loosestrife's beauty is deceptive. Native vegetation and animals that depend on wetlands disappear as those wetlands become dominated by loosestrife. A study conducted recently by our laboratory showed that early-season nesting by red-winged blackbirds (*Agelaius phoeniceus*) was nearly non-existent in several Illinois wetlands dominated by loosestrife; the birds shifted to loosestrife as a nesting substrate later in the season, so nesting was not prevented, merely delayed to the second brood (Maddox and Wiedenmann, 2005). The weed has become increasingly invasive in Illinois over the past 20 years or so, crowding out native vegetation in once-pristine sedge meadows, bogs and areas designated as nature preserves.

Biological Control: the Last Hope for Success

Land managers have battled loosestrife for decades, having little luck against vast stands of the weed. Biological control was seen as the last best hope to combat purple loosestrife. A project to search for natural enemies of loosestrife began in the 1980s, centred at the CABI laboratory in Delemont, Switzerland. Coordination of the project in North America was through a US Fish and Wildlife research unit located at Cornell University, but with numerous other partners. The project, eventually headed by Dr Bernd Blossey at Cornell, resulted in the selection and importation of several biological control agents approved for

release. Agents included two species of leaf beetles (*Galerucella californiensis* and *Galerucella pusilla*) (Fig. 11.1), a root-feeding weevil (*Hylobius transversovittatus*) and two flower-feeding weevils (*Nanophyes brevis* and *Nanophyes marmoratus*). For a review of the project and agents, see Malecki *et al.* (1993).

Releases of *Galerucella* species at limited numbers of sites began in the early 1990s, with greater distribution beginning in 1994 – the year that the programme in Illinois began. A group of state and county land managers in Illinois cobbled together \$14,000 to purchase (at \$2 per beetle) 7000 beetles, which were released at seven sites near Chicago, in northern Illinois. Although the land managers had experience battling loosestrife, none had a background in biological control. Two of us (RNW and DJV) attended a regional meeting on biological control of loosestrife late in 1994, after which we decided to develop a larger programme, with the support of entomologists and biological control specialists from the Illinois Natural History Survey (INHS). INHS is a research and outreach agency, having no responsibility for land management, and is located approximately 120 miles

(a)



(b)



Fig. 11.1. Adult and larval *Galerucella californiensis* on a purple loosestrife.

(210 km) from the loosestrife-infested wetlands in the Chicago area, where most of the wetland sites were located. A natural partnership developed – INHS had expertise in biological control and insect rearing, whereas the land managers managed the wetland sites.

A Biological Control Programme is Started in Illinois

With seed funding from several county Forest Preserve and Conservation Districts, we initiated our programme in spring 1995. We learned rearing techniques from Dr Blossey, and we began growing loosestrife plants in the greenhouse and rearing *Galerucella* on them. With a few missteps (particularly infection of the beetles by the fungus *Beauveria bassiana*, alleviated by greater air movement), we proudly produced and released 2300 *Galerucella* at seven sites in 1995.

During the winter of 1995–1996, our programme gained speed, as we planned for larger-scale releases in 1996. State and county land managers selected 30 release sites, from among hundreds possible, that they considered the most threatened and in need of control measures. Because the beetles are synchronized with early vegetative plant growth, we planned to release 60,000 beetles from May through early July, with at least 2000 beetles at each of the 30 sites. We taught the cooperators about biological control, insect biology and how to recognize adult and immature beetles feeding in the field. Finally, we filled a greenhouse room with over 500 caged loosestrife plants to use for producing the beetles.

Legal Battles Result in Support for Biological Control

Beetle production and releases in 1996 exceeded all expectations. Over 130,000 adult *Galerucella* were released at the 30 selected sites, which included several high-quality nature preserves. We found eggs and larvae from the releases made in 1994 and 1995, assuring us that the insects could establish. We also developed a significant partner in the Regulatory Branch of the Chicago District office of the US Army Corps of Engineers. That office, responsible for permits to developers, had jurisdiction (until a later court ruling) over wetlands in the Chicago region. Historically, Chicago was mostly a complex mosaic of wetlands, meaning that the Corps was responsible for reviewing nearly all development projects since obtaining its regulatory authority under the Clean Water Act in 1972. One requirement for getting a wetland permit from the Corps was an absence of any purple loosestrife at the site at conclusion of the project. This requirement proved nearly impossible in many cases, meaning the permit holder either didn't receive final compliance sign-off of their permits or fought the Corps in court over the final resolution of their permit conditions; this stand-off was costly and time consuming for both the developers and the Corps staff.

One insightful Corps project manager, Kathy Urquhart, realized that biological control offered what herbicides or other methods didn't – the possibility (not the promise) of controlling loosestrife at the permit sites under their jurisdiction. With our help, she developed a plan to offer the possibility of using

biological control for mitigation – developers bought insects from INHS, which we then released at their permit site containing loosestrife; thus INHS received the funding for rearing large numbers of insects. When the developers purchased the insects – at less cost than fighting in court or paying to treat sites with herbicide – the Corps signed off on their wetland permits, absolving the developers of further responsibility involving loosestrife for their mitigation site. Further, the Corps was able to close the files on numerous permit sites, thus freeing staff time to better review incoming project plans. It was viewed as a win-win-win situation for all parties.

For the next few years, funding from those mitigation projects allowed us to rear and distribute large numbers of insects, and expand our network of cooperators to other counties, city park districts and private foundations, as well as an increasing number of environmental consultants that worked as liaisons between the developers and the Corps. We shifted our methods to rear the beetles in large outdoor, walk-in cages, to ensure that the beetles were acclimated to ambient light and temperature conditions. That improvement and other refinements led to the production and release of nearly 2.7 million *Galerucella* at over 230 sites by 2005.

Signs of the beetles' impact against large populations of loosestrife began to occur in isolated sites as early as 1997 and dozens of sites by 2003. Some release sites showed a significant and sustained reduction of flowering loosestrife plants. At other release sites, there was a 'boom-and-bust' cycle. Severe defoliation of all loosestrife plants occurred at the site (some sites were many hectares in extent) followed by the movement of the *Galerucella* from the site as they sought additional food. This was then followed by a resurgence of loosestrife, and the subsequent return and build-up of *Galerucella* the next year.

Expanding the Programme to Young Scientists

The funding arranged through the Corps also allowed us to expand the project in a different way. INHS has had a history of strong outreach and public education. One of us (MRJ) is the Education and Outreach Coordinator for INHS, with significant experience translating science for the public, including developing curricula and classroom materials on a range of biological projects. In 1997, the four of us met to discuss the prospect of developing training materials and curricula for schools on biological control and purple loosestrife. Our interest was in high-school educators and their students. We started with materials previously developed to teach biodiversity, and developed a new curriculum, entitled 'Biodiversity, wetlands and purple loosestrife: information and activities for young scientists; Purple loosestrife: a case study.' This 225-page notebook was developed with a pyramid approach: the broad base contained information and exercises about biodiversity; the next level (more narrow) contained information and exercises about a specific habitat, wetlands; the next level, purple loosestrife as an invader of wetlands; and higher levels dealt with specifics about biological control as an approach to combating invasive weeds, biological control of purple loosestrife, and the capstone (action project) consisting of rearing *Galerucella* beetles and releasing them into nearby wetlands.

In concert with the curriculum notebook, we developed an all-day workshop to train educators in the use of the classroom materials and the 16 classroom activities contained in the notebook, with the major activity to be rearing beetles in the classroom. The activities were structured as investigative questions and procedures, but also included assessments of the activities, instructions on how to extend them, and the relation of the activities to state learning goals. The workshops were hands-on: immediately after registration, the educators set up their own host-specificity trial, which they observed periodically and assessed at the end of the workshop. During the first half of the workshop, the topics of biodiversity, wetlands and biological control were covered and activities (e.g. measuring diversity in a simulated wetland transect) and games (e.g. population dynamics) were demonstrated, and then tried by the educators. The second half of the workshop was devoted to the action project – how to grow purple loosestrife and rear the *Galerucella* beetles. Educators may have had a lot of interest and enthusiasm about the project, but most had little knowledge about growing plants in the classroom and handling insects. Educators were provided with everything needed to grow loosestrife and *Galerucella* beetles in their classrooms – from loosestrife roots and soil to Petri dishes and aspirators. Later in the spring we shipped, via overnight courier service, vials of beetles newly emerged from overwintering (overwintering was necessary for oviposition). Workshops were held at the end of January and beginning of February to allow sufficient time to grow the plants (~ 4 weeks), rear a new generation of beetles (~ 6 weeks) and make releases before the end of the school year. As a trial, we set up in our laboratory a simulation of the facilities for classroom rearing, ensuring that everything needed in classrooms was made available to the educators. In short, we didn't want the lack of any item to deter anyone from completing the project. We billed our programme as I² – immediately implementable, a quality which was affirmed in comments received from several educators.

The first year (1998), we trained 25 educators. Funding for the education project that year was provided by Chicago Wilderness, a consortium of environmentally active groups, institutions and public entities. That funding allowed us to provide all the materials to each educator, as well as ship adult beetles ready to oviposit. Costs of the rearing kits, curriculum materials and shipping beetles were estimated at approximately \$500 per classroom. This cost was greater than nearly any school could absorb, so our intent was to find the funds to be able to provide everything to all educators, regardless of their ability to fund the project.

Word of the project spread quickly, and in the second year we trained approximately 60 educators in three workshops; in subsequent years, we settled on two workshops per year, each of which could accommodate approximately 25–30 educators. Funding for the next few years was provided by Chicago Wilderness (once more), as well as by the US Fish and Wildlife Service, USDA-APHIS National Biological Control Institute, the Illinois Department of Natural Resources, and funds through the Army Corps mitigation projects. As of 2005, we had trained more than 350 educators, primarily classroom teachers but also some educators located at nature centres. We designed the curriculum for high-school teachers, but we found that educators of all-age children participated and adapted the materials for the appropriate age groups. We were

assuming the teachers needed age-appropriate materials, but we learned that they wanted *any* materials; they were skilled at adapting our materials for any age. In fact, we had teachers from classrooms ranging from Advanced Placement seniors in high school to kindergartners.

Comments from the educators have ranged from ‘this is one of the best classroom exercises we have done’ to ‘the children are so proud to be part of real science and real ecological help to a wetland’. Kindergarten teacher Laura Mendoza wrote, ‘Many happy connections are being made with some of the activities you’ve provided and through other things we’ve done. At the kindergarten level, I believe there is a good deal of thinking and awareness going on. Your project is intended for older children, but there is all of that basic underlying knowledge appropriate for these young minds.’

An Unexpected Partnership: the Public Embraces Biological Control

Students grew the plants, reared the beetles and released them in wetlands (Figs 11.2 and 11.3). Some of them began monitoring the populations of loosestrife and establishment of beetles. But another curious thing occurred – students went home and told their parents about this new activity they were doing at school. Many of those families lived in neighbourhoods or housing developments that included natural or created wetlands, which contained purple loosestrife. Those groups of homeowners, both informal groups and formal homeowner associations, contacted us at INHS and they too wanted to be involved in rearing beetles for the wetlands in their neighbourhoods, often using their own backyard (Fig. 11.4).



Fig. 11.2. High-school student inoculating her loosestrife plants with *Galerucella* beetles.

(a)



(b)



Fig. 11.3. High-school students taking their classroom-reared *Galerucella* beetles to a wetland and releasing them.

This was a new twist for us, the possibility of educating and supporting a totally new constituency.

As much as the homeowners had to learn, we had to learn even more. We were no longer necessarily dealing with people who already knew something

(a)



(b)



Fig. 11.4. Homeowners rearing *Galerucella* in their backyards. (Photos by Jack and Bev Mompson.)

about biology, as our educators had. Instead, our new partners were investment bankers, chemists, retired engineers, 4-H leaders, scout groups and homemakers. Concepts such as growth and metamorphosis, life cycle, diapause, phenology, oviposition and host specificity of biological control agents were totally foreign to this group. Partnering with this new constituency required us to develop support materials that took nothing for granted. They didn't need (or want) the detailed notebook; instead, they wanted distilled, bare bones how-to guides and materials, especially with illustrations. We developed brief, laminated, photo-filled,

two-sided guides to planting roots, growing beetles, releasing beetles, recognizing feeding by *Galerucella* (as opposed to occasional inconsequential damage from mirids). We adapted our all-day workshop for educators to a half-day hands-on demonstration for homeowners, usually held about the end of April (purple loosestrife begins growing about that time in northern Illinois) (Figs 11.5 and 11.6).



Fig. 11.5. One of the many Illinois cooperators releasing *Galerucella* beetles into a loosestrife-infested wetland.



Fig. 11.6. Cooperators and partners at a field day, where *Galerucella* are showing promise.

Lessons Learned from Engaging the Public

One question asked by other states that wanted to begin a programme like this was, 'how do you do it?' Rearing insects and growing plants were second nature to us, but to educators and homeowners, these processes were often completely foreign and generated innumerable questions. We used several methods to keep in touch with everyone, answer the questions and troubleshoot problems (SLP handled most of this). The most-used tool was the 'beetle hotline', a lab phone number with voice mail. We routinely received messages time-stamped at 1:30 am, 2:30 am, by people who were surprised that there was not a staff member working overnight to answer their questions live! Most calls and questions were answered within 24 hours, if not immediately. We were always in touch with our partners, wondering when they could take a shipment of beetles and asking them what they were seeing in the field. Educators received stamped postcards to send back to us, to let us know how their plants were doing and if they had any questions. Questionnaires were sent out at the beginning of the school year to find out if the educators had any problems, any suggestions for improving the programme and if they would be participating again next year. Homeowners received phone calls asking when they were ready for insects and if they had any questions. We also promoted our project with purple loosestrife pens, magnets, stickers, hats and t-shirts.

Engaging the public in a specialized project such as this was not without its pitfalls. We trained a significant number of educators who extended the research and rearing into their classroom. However, not all teachers were as well prepared (or as motivated) as we might have liked. One teacher didn't use the lights but instead tried growing her plants in a dark boiler room – warm enough but no light. The plants died. We could likewise take nothing for granted with the homeowners. One homeowner showed us her potted loosestrife plants that had died. How do you kill loosestrife plants? After all, they are weeds! Well, she had potted the roots upside down. At the next workshop, we invoked the saying 'green side up', half in jest – but only half. Each year we received phone calls from distraught homeowners (about a month after inoculating their plants with beetles) telling us they did something wrong, because their plants were brown and looked dead. We told them to wait 2–3 more days and then call us back. The plants were 'brown and looked dead' because they had been defoliated, and 2–3 days later they had abundant, newly emerged adult *Galerucella*.

Certainly many educators didn't continue after their first year, for one reason or another. We know the project worked, however, because we had some educators participate for 6 years and more. We also had to recognize that whatever we did, whatever support we provided, was not going to resonate with everyone. If we had taken a few drop-outs as a sign of failure we would have given up. Instead, we found solace in those educators who persisted, and who taught us a few things that we adopted for later participants who really wanted to learn and be a part of the project. Those people energized us and kept us going. All groups required continued significant support, and in unique ways. Rather than be frustrated by that, we learned a few lessons ourselves. We were continually amazed at the amount of support and input that was expected of us.

The Many Benefits of Involving the Public

We believe the goal of employing broad public participation worked. Several examples illustrate this. Two aspects of this classroom project were most important to us: (i) students were learning ecology by participating in the project, rather than reading about it in a textbook; and (ii) many of the educators participated in the project each year; in fact, approximately 125 of the 350 were still (2005) participating annually, including a number of them from the first workshop in 1998.

Some educators have taken this project to levels we never imagined. One educator found a site that consisted of two small islands in a lake, separated by a few hundred metres. Her class released *Galerucella* on one island but monitored loosestrife on both islands, both to document differential changes and also to see if and when the beetles found their way to the other island. Other educators developed new monitoring techniques, which worked far better than we had envisioned, such as collecting flower heads in the autumn (when classes resumed) to measure changes due to feeding by *Galerucella*. One educator in our first workshop had a student in her class that later attended the University of Illinois, majoring in Elementary Education. By chance, this student applied for an hourly position we had available in our lab; only after we hired her did she tell us she had worked on the project as a student. Even better, she later adapted the project for use in her student teaching and her future job as an elementary teacher. Another student took the classroom materials and adapted them for use in her own wetland (before we developed materials for homeowners), and won first place at the State Fair. Another used loosestrife biocontrol for his Eagle Scout Project. Finally, one homeowner couple was awarded the 'Citizen Volunteer of the Year' Award from the Illinois Department of Natural Resources.

Because the homeowners, 4-Hers and scout groups could rear the insects outdoors (as opposed to most of the educators), they also were able to rear and release larger numbers of beetles. The numbers of homeowners growing plants and beetles in mesh cages peaked in 2002 with 12 groups and 234 cages of beetles. Although beetle production in classrooms was minimal (it was largely for demonstration and participation), some of the homeowner cages produced more than a thousand adult beetles each. Thus, the number of insects produced by homeowners significantly added to our efforts, as well as providing new sites for releases.

Does it work to have Everyman participate in such a biological control project? Certainly it depends on the project. In the case of *Galerucella* and purple loosestrife, the rearing methods were adaptable to classroom and backyard settings. The feeding by chrysomelid beetles provided visible evidence, which was convincing (and rewarding!) for homeowners and schoolchildren. Other loosestrife agents, particularly the *Hylobius* root weevils, would not have been as amenable – they have long life cycles, and very cryptic disposition and behaviour. The support necessary for either classroom educators or homeowners cannot be minimized. Specialized materials, availability by phone or e-mail, and even personal visits to classes or homeowner sites often meant the difference between participation and the lack thereof.

The costs can be great (even beyond the \$500 per classroom we spent), but we believe the benefits exceed the costs. We developed a network of informed citizens that have a basic understanding of what biological control is and why it can be used against invasive weeds. Those people are not only aware but they are also vocal, telling friends and neighbours, and also local politicians. Developing a new generation of scientifically literate citizens will pay dividends long into the future. At a time when the misunderstanding of science and the scientific process is rampant (one well-educated physician mistook biological control agents for biological warfare agents, such as anthrax), anything that demystifies the process is a benefit to us all. Further, support for our institutions (in our case, specifically INHS) is crucial in times of shrinking budgets. Being relevant to the public – with a project that affects their own neighbourhood – carries great weight.

Finally, one never knows whom we may educate. One of our release sites was on the south side of Chicago, an area notorious (perhaps undeservedly) for violence and drug traffic. Police seeing a stopped vehicle usually have cause for concern or inquisitiveness. One spring, we stopped at this site to look for emergence of overwintered beetles. A patrol car from the Chicago Police Department pulled up and stopped. Two burly officers emerged and approached us, wondering what we were doing at the edge of the wetland in this neighbourhood. As we started to explain about the project, one of the officers exclaimed (probably with relief), 'Oh! You're the beetle guys!' And they took the time to look around the wetland to find our beetles. One never knows just whom one can reach.

So, did Everyman find his friends on the path, sustained by 'Good Deeds'? In our project, we can give a resounding 'yes'. The journey has been long, sometimes hard, but more often rewarding. Having the public help give us a 'strait count' extended our efforts far beyond what we were capable of achieving alone. More importantly, we have paved the way for the public to understand, support, and participate in future projects.

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12

Biological Control for Insect Pests in Greenhouses: an Unexpected Success

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Overview: Crop protection in greenhouses became strongly chemically oriented in the 1950s. Rapid development of pesticide resistance initiated a search for alternatives. Presently, in greenhouse vegetables of northern Europe, most arthropod problems can be solved without the use of pesticides. This is the story of the 30-year evolution in control practices that has led to the implementation of biological control as the primary pest management practice in greenhouses.

Introduction

In this chapter I intend to show how successful biological control programmes were developed for greenhouse production systems. When we started with the development of the first greenhouse biological control programme in the 1970s, I initially failed badly. I still vividly remember our first contacts with growers when talking about using the parasitoid *Encarsia formosa* for control of whitefly. We had worked for quite some time in the laboratory to study the relationship between whitefly and the parasitoid. Based on this work, I concluded that this parasitoid might be a good candidate for whitefly control, and could solve the problems associated with pesticide resistance in whitefly. When proposing to growers to release *Encarsia*, their reaction was ‘do you think that we are going to release even more insects in our greenhouses; no way, we have enough insect problems already.’ My face turned red; I was embarrassed and disappointed because I thought I was proposing a beautiful solution. But I had neglected a number of important points. First, knowledge about biological control had almost completely disappeared among growers during the 25 years of pesticide use. Secondly, costs for chemical pest control amounted to less than 2% of the total production costs of vegetables in greenhouses, and growers will not run a risk with a new method if they are not absolutely sure that it works as well as chemical control for the same low price. Thirdly, I had a solution for only one pest, and this parasitoid would not be

able to function in a greenhouse where the grower has to spray against another pest. So for all pest and disease problems we had to find solutions that were compatible with biological control. This took a while, demanded a group effort and much coordination, but was very satisfying (van Lenteren and Woets, 1988). I will describe the lessons we learned and the solutions that were found, and I will summarize how much the attitude of growers concerning pest management in greenhouses has changed.

Lesson 1. Discuss the Development of a New Crop Protection Method with All Stakeholders

Nowadays, when a new pest or disease is perceived, we organize a meeting with all stakeholders (growers, pest control specialists, extension service, researchers (e.g. plant breeders, entomologists), etc.) and discuss potential short- and long-term solutions. The initiative for such a meeting can be from any group of stakeholders, and we follow the 'joint analysis and decision making process' (C. Leeuwis, Wageningen, 2005, personal communication). The conclusion of such a meeting might be that biological control is the best solution. At that same meeting we then discuss what other conditions need to be fulfilled for the proper functioning of biological control in the greenhouse setting. A major point is always that a complete pest and disease management programme should be available, in which natural enemies can function. If one of the chemical pesticides used for arthropod, disease or weed control has a negative side effect on the new natural enemy, then biological control is not realistic until an alternative for this pesticide has been found (van Lenteren, 1995).

Sometimes we have to do specific research to find such an alternative, but we can also profit from old information (= pre-1950 pest control) and from results presented during IOBC working-group meetings (see below). Until 1950, integrated pest management was not recognized as such. Organic pesticides were hardly available and many different control techniques were combined. Cultural control, host-plant resistance and biological control were important aspects of pest management in greenhouses. Interest in integrated control developed shortly after the appearance of synthetic pesticides after 1940, owing to the development of resistance to chemical controls and the recognition of unwanted side effects. This led to the formation of the International Organization for Biological Control (IOBC; www.IOBC-Global.org) in 1955. The European section of the IOBC has been the driving force behind a change of thinking in crop protection ever since, and has coordinated many cooperative biological control and IPM projects, including those in greenhouses (van Lenteren *et al.*, 1992). The often very time-consuming development of these IPM programmes was only possible because of intensive cooperation within the IOBC Working Group Integrated Pest Control in Greenhouses (see www.IOBC-WPRS.org under working groups for activities and publications).

Information from the above sources is used to design a draft biological control programme for the new pest or disease, including an overall IPM programme for the other pests and diseases. This is then discussed in following meetings with the stakeholders until agreement has been reached about the applicability

of the programme. In the next phase, the IPM programme is implemented, further developed and may even lead to production systems where pesticides are no longer needed (van Lenteren, 2000).

Lesson 2. Work with the Best, Most Progressive Growers when Developing Biological Control

Very early in the development of the first biological control programmes, we learned that it is crucial to cooperate with the most progressive growers. To our initial surprise, they were very interested, took up the knowledge very quickly, suggested many improvements, saw possibilities to advertise products produced under biological control, and they were able to convince other growers how useful biological control was. It was these growers who allowed us to do experiments in their commercial greenhouses, and who invited other growers and the extension service to demonstrate how well biological control worked. We could not have found better advocates for biological control!

Lesson 3. Develop Good Teaching Material about Biological Control; Retrain the Extension Service and Growers

When we experienced that modern growers in the 1970s had never heard about biological control, our conclusion was to develop teaching material for vocational schools, high schools and universities. A fruitful coincidence was that this happened during a period when many persons were concerned about environmental problems. Teachers of science and biology were very happy that they could link the development of an applied ecological method that was beneficial for the environment to general biological issues. Also, they could easily do experiments with commercially available natural enemies to illustrate population dynamics and the existence of trophic levels in ecosystems. The result was that teaching of biological control took off very quickly and had a clear impact on changes in thinking about crop protection: children and students taught their parents how biological control worked.

We also realized that it was necessary to retrain the extension service personnel. Some had taken the initiative to be informed; others were rather sceptical about biological control and hampered its implementation. It became clear that biological control took off much better in areas where the extension service workers were enthusiastic. Next, and often together with the extension service, we organized free courses on biological control during the winter to train the farmers in recognizing the natural enemies and pests, and in sampling and release methods.

In addition to training, we published in journals that the growers use primarily for obtaining the newest information on production techniques. So next to our scientific publications, we intentionally and regularly wrote extension papers. We also kept an eye on sources that the growers often use to collect pest control information and made sure that material about biological control was available there (van Lenteren, 1990).

Lesson 4. Provide/Sell Pest Management Guidance and Not Only the Biological Control Agents

The danger of just providing natural enemies is that if they do not work, the grower is disappointed and will speak negatively about biological control. A producer of natural enemies in the Netherlands, who was earlier a grower of greenhouse cucumber, understood this problem and together we developed a guidance and information system, which is sold to the growers for a certain price and includes provision of the natural enemies. A member of the personnel of this natural-enemy producer visits growers and decides when and how many natural enemies the grower should release. During subsequent visits, he samples and decides whether the natural enemies are working, or if more releases are need. This person also advises about IPM and which chemical pesticides might be integrated with the natural enemies. Nowadays, this system is still in force, although the information about side effects on natural enemies can also be found on websites of natural-enemy producers (e.g. www.biobest.be and www.koppert.com).

Lesson 5. Develop Effective, Economic and High-quality Mass Production of Natural Enemies

Two small producers were active in 1968, when commercial biological control in greenhouses started in Europe. Today, Europe has more than 30 commercial natural-enemy producers including the world's three largest. The three largest serve more than 75% of the greenhouse biological control market worldwide. Of the more than 100 biological control agents that are marketed for pest control in greenhouses, about 30 make up 90% of the total sales (van Lenteren, 2003). It appears that many more species of biological control agents are available in Europe than elsewhere (van Lenteren *et al.*, 1997). This is owing to the much larger greenhouse industry and a longer history of research in greenhouse biological control in Europe.

Mass production of natural enemies has seen a very fast development during the past three decades. The numbers produced have greatly increased (up to 50 million individuals per week), the spectrum of species available has widened dramatically (from two in 1970 to more than 100 nowadays), and mass-production methods clearly have evolved (van Lenteren, 2003).

Companies starting the production of natural enemies usually have little knowledge about the obstacles and complications related to mass rearing. They are even more ignorant about the development and application of quality control. A special point of concern is the lack of knowledge about the sources of variability of natural-enemy behaviour and methods to prevent genetic deterioration. This may result in natural enemies of bad quality and failures of biological control programmes. The few large companies have entomologists employed who developed quality-control tests, but methods differ widely and are not always adequate (van Lenteren, 2003). And even when the natural enemies leave the insectary in top condition, it does not mean that they are in top shape when

released in the greenhouse. Shipment and handling by the producers, distributors and growers may result in deterioration of the biological control agents. This makes robust quality-control programmes a necessity (van Lenteren and Tommasini, 2003).

In the 1990s, commercial producers of biological control agents and scientists started to work on development and standardization of quality-control methods. Quality-control procedures for natural enemies were developed and published for the 30 most important species of natural enemies commercially applied in greenhouses (van Lenteren *et al.*, 2003). Quality-control criteria relate to product control and are based on laboratory measurements, which are often easy to carry out. The criteria will soon be complemented with flight tests and field performance tests. Research and application of quality control is coordinated by a working group of IOBC (see www.IOBC-Global.org and go to the working group Arthropod Mass Rearing and Quality Control (AMRQC)).

Lesson 6. Be Realistic: Not All Pests Can Be Managed with Biological Control

Pushing for biological control as the only solution to control pests is a utopia. Biocontrol workers need perseverance, green fingers and creativity, but sometimes biocontrol is not the best solution or will not work. An example is pest control in short-term crops, like lettuce. In Holland, lettuce is produced during 6-week cycles and one of the main pests is aphids, a notoriously quick-developing pest, which is difficult to control, also in long-term crops. We were able to keep pests under biological control with frequent releases of great numbers of a whole array of natural enemies, but it was too expensive to be of practical use. Here we had to conclude that development of host-plant resistance to aphids was the first important step, and when this was realized and became a success, we could advise biocontrol for other pests, such as leaf miners (De Ponti and Mollema, 1992).

Lesson 7. Expect the Unexpected . . . Recover and Win

A dramatic experience was the development of pyrethroids in the 1980s. After 15 years of using successful biological control programmes for the main vegetable crops in greenhouses (van Lenteren and Woets, 1988), pyrethroids appeared on the market and were effective against a number of important pests, making biological control redundant. The result of their use was that the biological control producers almost went bankrupt. 'Luckily' enough, some pest species quickly developed resistance against these pesticides, and the help of the old natural enemies was needed again. We learned an important lesson: it is crucial to know the effects of a new pest control agent on natural enemies in an already perfectly working IPM programme. Initially, side effects of pesticides on natural enemies were tested within an IOBC working group. The work of this group resulted in a very important development in pesticide legislation within the European Union: the applicant

of a new pesticide now has to provide data about safety of the pesticide for natural enemies. Such data can then be used to advise for or against the use of this pesticide within an IPM programme. Several side-effect lists have been published, as well as testing methods (see www.IOBC-WPRS.org, and go to working group Side Effects of Pesticides on Natural Enemies). Also some producers of natural enemies have these lists on their websites (see last line of Lesson 4) so that growers can quickly check whether a pesticide can safely be used in an IPM programme.

Lesson 8. With Good Support and Guidance, Growers Prefer Biological Control as First Option for Pest Management

After initial hesitation, growers are now using biological control on a large scale. We received the following responses when we asked them if and why they prefer biological control.

1. With biological control there are no phytotoxic effects on young plants, and premature abortion of flowers and fruit does not occur; also yield increases have been obtained with biological control.
2. Release of natural enemies takes less time and is more pleasant than applying chemicals in humid and warm greenhouses.
3. Release of natural enemies usually occurs shortly after the planting period, when the grower has sufficient time to check for successful development of natural enemies; thereafter the system is reliable for months with only occasional checks. Chemical control requires continuous attention.
4. Chemical control of some of the key pests is difficult or impossible because of pesticide resistance.
5. With biological control there is no safety period between application and harvesting fruit; with chemical control one has to wait several days before harvesting is allowed again.
6. Biological control is permanent: once a good natural enemy – always a good natural enemy.
7. Biological control is appreciated by the general public; they have more respect for our work, and sometimes we receive a better price for the product.

These advantages are so important for growers that they will not easily return to chemical control. Today it is not a matter of using biological control or not, but how many species of natural enemies they will release.

Lesson 9. With Most Arthropods under Biological Control, Concentrate on Biological Control of Diseases

Until a few years ago biological control was limited mainly to control of arthropods. However, disease problems can be considerable, particularly in tomatoes, cucumbers and cut flowers. Some fungicides can be integrated with the use of

natural enemies, but as problems of fungicide resistance are strongly increasing, fewer 'relatively safe' fungicides remain available. Thus, serious negative effects of fungicides on natural enemies of insects and widespread resistance of foliar pathogens to fungicides led to demands for alternatives (see Jarvis *et al.*, Chapter 25 this volume). Although use of fungicides remains substantial for foliar pathogens, disease management is now evolving towards use of resistant cultivars and manipulation of the environment. During the past decade, several initiatives led to research in non-chemical control, such as the effect of soil solarization on nematodes and fungi, and the potential use of antagonistic leaf fungi (Albajes *et al.*, 1999). For an overview of recent successes and practical applications with disease-suppressive soils, biological control of soil-borne pathogens, and root, stem or foliar diseases, I refer to van Lenteren (2000). Nowadays more than ten microbial products are registered and used for pest and disease control in greenhouse vegetables and ornamentals in Europe, and about five bacterial and fungal products for control of fungi are in the last phase of the registration procedure.

Lesson 10. Do Not Be Afraid to Approach Seemingly Impossible Situations: the Case of Biological Control in Brazilian Greenhouses

Protected cultivation is a relatively new production method in Brazil. The first initiatives for greenhouse production of flowers took place in Holambra (State of São Paulo) in the 1970s. Currently there are about 1400 ha under protected cultivation in Brazil. Pest management is still mainly by chemical pesticides, and 2–3 sprays per week during the whole production season are no exception. Therefore, a research programme on biological and integrated pest control for greenhouse pests of a major crop, chrysanthemum, was initiated in 1998 between a commercial ornamental producer and the Department of Entomology of Lavras University (Brazil), with collaboration of the Laboratory of Entomology, Wageningen University (the Netherlands).

The first phase of the project consisted of a comparison of the presence and development of mainly aphid pests on sprayed and unsprayed chrysanthemum crops. Spraying was according to normal practices in commercial greenhouses. In the chemically controlled crop, the spray frequency was on average 2–3 times per week, and aphid densities remained below the economic injury level. In the unsprayed crop four aphid species were found. The same aphid species were present in both greenhouses, but in the unsprayed greenhouse three species of parasitoids and several species of predators were also found, which had spontaneously immigrated and were not killed by the pesticide. Aphid densities in the unsprayed crop never reached the economic injury level. Also, the densities of other pest organisms (leaf miners, whiteflies, spider mites and thrips) stayed well below the economic injury level. The end result of the study in this first season was both surprising and satisfactory: the number and quality of flowers produced in the unsprayed chrysanthemum crop was the same as in the chemically controlled chrysanthemums (Bueno *et al.*, 2003).

During the second phase, the development of more pests and natural enemies was followed, again in a sprayed and unsprayed chrysanthemum crop. Aphids, thrips and whiteflies were observed, together with species of natural enemies, including specialist parasitoids and predators. All these natural enemies spontaneously entered the greenhouse. When we compared pest densities in sprayed and unsprayed crops, we could draw the following conclusions:

- Thrips and whitefly numbers were lower in the unsprayed crop than in the sprayed crop, but numbers in both crops did not cross the economic injury level.
- Aphid numbers were higher in the unsprayed crop than in the sprayed crop, but numbers never crossed the economic injury level.
- Parasitoid numbers were much higher in the unsprayed than in the sprayed crop.
- Predator numbers were higher in the sprayed than in the unsprayed crop.

So, none of the typical pests in chrysanthemum caused problems in the unsprayed greenhouse. In this greenhouse, natural enemies spontaneously invaded and provided efficient control that is free of charge. It is interesting to note that more predators were observed in the sprayed greenhouse than in the unsprayed one. This might be caused by a combination of a lower sensitivity to pesticides of predators compared with parasitoids, and a more generalist nature of predators concerning prey choice compared with more specific parasitoids. Also, in this second year, the flower yields in the unsprayed greenhouse were as good as in the sprayed one. These results surprised the growers, and after this second year of good results, they started to accept the idea that biological control can be a reliable way of pest management. An economic evaluation of improved profits in unsprayed chrysanthemum crops is now urgently needed.

At the start of the third phase of the project, the growers were so enthusiastic about the results that they offered to build a special greenhouse for biological control research. We have set the following goals for the third phase: (i) follow development of pests and natural enemies in the new, pesticide-free greenhouse and compare it with data from conventionally sprayed greenhouses; (ii) develop an open rearing system for aphid control, with parasitoids able to correct for unexpected aphid invasions and development; and (iii) release an aphid parasitoid and a thrips predator in low numbers to have natural enemies in the production system ready for control of these pests when their numbers might come close to the economic threshold density. The goal of the third phase is to develop a reliable biological control method based on a combination of spontaneously immigrating natural enemies and augmentative releases (Bueno *et al.*, 2003).

Current Situation in Greenhouse Biological Control

Biological control is used on a large scale in all main vegetable crops. In The Netherlands, for example, more than 90% of all tomatoes, cucumbers and sweet peppers are produced under IPM. Worldwide 5% of the greenhouse area is

under biological control, and there is potential for increase to about 20% of this area in the coming 10 years (see also Shipp *et al.*, Chapter 13 this volume).

A good example of a biological control in an IPM programme is the one for tomato in Europe. It involves ten natural enemies and several other control methods, such as host-plant resistance, climate control and cultural control (Table 12.1). At a first glance, such an IPM programme may look complicated, but after a year of experience and support from the provider of biocontrol agents, most growers are able to carry it out. A recent development which gave a strong stimulus to the

Table 12.1. Biological control in the Integrated Pest and Disease Mangement programme as applied in tomato in Europe (after van Lenteren, 2000).

Pests and diseases	Method used to prevent or control pest/disease
Pests	Control method
Whiteflies (<i>Bemisia tabaci</i> , <i>Trialeurodes vaporariorum</i>)	parasitoids <i>Encarsia</i> , <i>Eretmocerus</i> predators <i>Macrolophus</i> pathogens <i>Verticillium</i> , <i>Paecilomyces</i> , <i>Aschersonia</i>
Spider mite (<i>Tetranychus urticae</i>)	predator <i>Phytoseiulus</i>
Leaf miners (<i>Liriomyza bryoniae</i> , <i>L. trifolii</i> and <i>L. huidobrensis</i>)	parasitoids <i>Dacnusa</i> , <i>Diglyphus</i> and <i>Opius</i> , and natural control ¹
Lepidoptera (e.g. <i>Chrysodeixis</i> <i>chalcites</i> , <i>Lacanobia oleracea</i> , <i>Spodoptera littoralis</i>)	parasitoids <i>Trichogramma</i> pathogens <i>Bacillus thuringiensis</i>
Aphids (e.g. <i>Myzus persicae</i> , <i>Aphis</i> <i>gossypii</i> , <i>Macrosiphum euphorbiae</i>)	parasitoids <i>Aphidius</i> , <i>Aphelinus</i> predators <i>Aphidoletes</i> and natural control ¹
Nematodes (e.g. <i>Meloidogyne</i> spp.)	resistant and tolerant cultivars, soilless culture
Diseases	
Grey mould (<i>Botrytis cinerea</i>)	climate management, mechanical control and selective fungicides
Leaf mould (<i>Fulvia</i> = <i>Cladosporium</i>)	resistant cultivars, climate management
Mildew (<i>Oidium lycopersicon</i>)	selective fungicides
Fusarium wilt (<i>Fusarium oxysporum</i> <i>lycopersici</i>)	resistant cultivars, soilless culture
Fusarium root rot (<i>Fusarium</i> <i>oxysporum radicle-lycopersici</i>)	resistant cultivars, soilless culture, hygiene
Verticillium wilt (<i>Verticillium dahliae</i>)	pathogen-free seed, tolerant cultivars, climate control, soilless culture
Bacterial canker (<i>Clavibacter</i> <i>michiganensis</i>)	pathogen-free seed, soilless culture
Several viral diseases	resistant cultivars, soil-less culture, hygiene, weed management, vector control
Pollination	bumblebees or bees

¹Natural control: natural enemies spontaneously immigrate into greenhouse and control pests.

application of biological control is the use of bumblebees for pollination, because chemical control can no longer be used as it kills the pollinators (van Lenteren, 1995).

IPM programmes for cucumber, sweet pepper and aubergine are somewhat more complicated than the one for tomato, mainly because of a richer pest and disease spectrum, and therefore more natural-enemy species are needed. Detailed examples of IPM programmes for vegetables used in different parts of the world are presented in Albajes *et al.* (1999). Until 1980, biological and integrated control of pests was almost exclusively applied in tomato and cucumber, which are by far the largest vegetable crops. Today, IPM is being used in other important vegetable crops such as sweet pepper, aubergine, melon, strawberries and even sometimes in leaf vegetables like lettuce (Albajes *et al.*, 1999).

Development of biological control for ornamentals is even more complicated than for vegetables. The first problem is that many different species and cultivars of ornamentals are grown. In Western Europe, for example, more than 100 species of cut flowers and 300 species of potted plants are cultivated, and for several ornamentals more than 100 cultivars are produced. Other problems for implementation of biological control in ornamentals are that: (i) more pesticides are available than for vegetables and higher residue levels are accepted; (ii) the whole plant is marketed, instead of only the fruit, so no leaf damage is allowed; and (iii) a zero-tolerance is applied to export material. But, since the 1990s use of biological control is steadily growing in cut flowers (e.g. gerbera, orchids, rose and chrysanthemum) and potted plants (e.g. anthurium, poinsettia) (Parrella *et al.*, 1999). In gerberas, the developments have been particularly fast, and natural enemies were used on about 80% of the Dutch gerbera area in 1998 (W. Ravensberg, Berkel and Rodenrijs, 1999, personal communication). Biological control was applied on more than 10% (600 ha) of the total greenhouse area planted with flowers and ornamentals in 1998 in the Netherlands. Commercially used IPM programmes for ornamental crops are presented in Parrella *et al.* (1999) for chrysanthemum, in van Lenteren (1995) for gerbera, and in Gullino and Wardlaw (1999) for various ornamentals. Worldwide, I estimate that about 1000 ha of ornamentals are under biological control.

The Future of Biological Control in Greenhouses

In the 1970s, few specialists in biological control anticipated being able to employ natural enemies in greenhouses, because growing vegetables and ornamentals in this protected situation is very expensive and pest damage is not tolerated. This means that the usually well-trained, intelligent greenhouse growers will not run the risk of any damage from insects, just because of ideological reasons such as reduced environmental side effects compared to chemical control. If chemical control works better, they will certainly use it. Yet despite chemical control being comparatively simple and inexpensive, adoption of biological control has been remarkably quick in greenhouses, first in north-western Europe (van Lenteren and Woets, 1988; van Lenteren, 2000) and later in other greenhouse areas (Albajes *et al.*, 1999; Parrella *et al.*, 1999; see also Shipp *et al.*, Chapter 13 this volume).

The growers now clearly see the specific advantages of biological control in greenhouses.

This success has occurred primarily as a result of outstanding cooperation between research, extension, growers and producers of natural enemies, often within the framework of IOBC. Several current trends will lead to a strong increase in the application of biological and integrated control of pests and diseases in greenhouses. First, fewer new insecticides are becoming available because of skyrocketing costs for development and registration, particularly for the relatively small greenhouse market. Secondly, pests continue to develop resistance to any type of pesticide, a problem particularly prevalent in greenhouses, where intensive management and repeated pesticide applications exert strong selective pressure on pest organisms (see also Janmaat, Chapter 19 this volume). Thirdly, there is a strong demand from the general public (and in an increasing number of countries also from governments) to reduce the use of pesticides. Finally, in order to escape from the 'pesticide treadmill', more sustainable forms of pest and disease control will have to be developed (Lewis *et al.*, 1997).

For the greenhouse sector, several approaches are followed to develop sustainable production systems. Worldwide, the search for biological control agents of diseases is a high priority (e.g. Dik *et al.*, 1998; Jarvis *et al.*, Chapter 25 this volume). Next, a continuous search for and evaluation of natural enemies (parasitoids, predators and pathogens) of insect and mite pests takes place, either to improve control of current pests or to develop control of new pests (Albajes *et al.*, 1999; van Lenteren, 2000). Also, increased activity in resistance breeding to pests and diseases for greenhouse crops is taking place; about 30% of all breeding activities of important greenhouse breeding companies are now spent on resistance breeding (A. Poolman and De Lier, 2000, personal communication). In addition, partial resistance can often (but not always) be used in combination with biological control to obtain sufficient control. Further, plant breeders and biological control researchers have joined forces to develop plant cultivars which help natural enemies to perform better. An example is the research on cucumber cultivars, where lines with fewer hairs were selected, which resulted in a higher search efficiency of the parasitoid *E. formosa* and more parasitized whiteflies (van Lenteren *et al.*, 1995). Another area where plant breeders and biological control workers can mutually benefit is in that of chemical communication between plant, herbivores (pests) and natural enemies. It is well known now that several crops start to produce volatile chemicals after being attacked by a pest insect or mite (Dicke, 1999). These chemicals are used by natural enemies to detect infested plants. Cultivars of the same plant species show large variation in the amount of volatiles produced after attack. Selection and use of plant cultivars that produce higher amounts of natural-enemy-attracting volatiles may improve biological control.

Several expert systems, or decision-support systems, are being developed for pest diagnosis and integrated control. An important factor favouring the use of such systems in the greenhouse industry is the fact that this sector is technologically highly advanced, with widespread use of computerized control of environmental conditions (Parrella *et al.*, 1999). Recently developed expert systems in this field have included pest and diseases diagnosis, integrated management in specific crops, information on natural-enemy-release programmes and data

on side effects of pesticides on natural enemies (Shipp and Clarke, 1999). This type of decision-support system helps growers manage increasingly complex production systems. The continuous updating of decision-support software packages, which is an essential element, is still problematic, however. Also, models have been developed that simulate the local searching and parasitization behaviour of individual parasitoids in a pest-infested crop (van Lenteren and van Roermund, 1999). Such models can be used to understand pest–natural enemy dynamics and/or to adapt introduction schemes of natural enemies.

Because of the political and consumer desire to reduce pesticide use, the future role of biological and integrated control is expected to increase. This is aided by the extensive demonstration of its positive role and because many new natural enemy species still await discovery (see, for example, Gillespie *et al.*, Chapter 14 this volume). Various cost–benefit analyses have shown that biological control is the most cost-effective control method. With improved methods for evaluation of beneficial arthropods, an increased insight into the functioning of natural enemies, and more efficient mass-production methods, the cost effectiveness of biological control may even be increased. Together with other control methods, such as mechanical and physical control, kairomonal control, and host-plant resistance, new IPM programmes will be developed. During the first decade of this century, a greenhouse without conventional chemical pesticides could become a fact!

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13

From Chemical to Biological Control in Canadian Greenhouse Crops

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Overview: By creation of excellent partnerships that brought together Canadian researchers, biological control companies and greenhouse grower organizations, the Canadian greenhouse industry has almost completely transitioned from total chemical control to a balance of biological control and integrated pest management for managing pests of greenhouse vegetables. In temperate climates, greenhouses provide the ideal setting for the implementation of biological control since they are enclosed structures with sophisticated crop production systems. The result is a stable growing environment that can be manipulated to maintain the most favourable conditions for the biological control agents and to reduce invasions of pests and diseases. This history describes how the Canadian greenhouse vegetable industry went from only chemical control to biological control as the primary strategy for greenhouse pest management.

The Beginning of Biological Control in Greenhouse Crops

Until the mid-1970s pesticides were the primary, if not the only, control measure for pests and diseases of greenhouse crops in Canada and elsewhere (see van Lenteren, Chapter 12 this volume). Pesticides were relatively inexpensive, readily available and control was fast acting. In contrast, biological control agents (BCAs) were very expensive, not readily available, and efficacy was unreliable. At this point greenhouse cultivation was in soil and in structures that were small in size (0.1–0.5 ha) and with a low gutter height (2–2.5 m). All these conditions favoured the development of high plant disease pressures. Climate control was manual and strict sanitation was seldom practised. Compared to today's production practices, conditions in the past were at best 'primitive'. All these factors

were conducive to rapid and catastrophic outbreaks of pests and diseases, which needed to be controlled quickly to prevent extensive crop damage.

Observant growers and researchers in the 1920s noticed that a parasitic wasp (*Encarsia formosa*), when present in greenhouses, attacked and reduced the populations of the greenhouse whitefly, a major pest of tomatoes. By 1928, the pest control potential of this parasitic wasp was realized and mass production of the insect began in Canada (McLeod, 1939). As a result of this work, more than 18 million *E. formosa* were shipped to Canadian greenhouse growers from 1938 to 1954. It was reported that ‘. . . parasites had been sent by railway and ship to Newfoundland as well as by rail to all provinces of the Dominion.’ Unfortunately, with the development and success of DDT and other new pesticides in the 1940s, the use of these parasitoids was discontinued by 1945.

In the 1970s, the situation changed again, this time in favour of biological control. Widespread pesticide resistance developed in whiteflies and two-spotted spider mites, another major pest of greenhouse vegetables. Crop losses were so serious that by 1978 tomato growers were applying pesticides weekly and yet still faced whitefly numbers so high that pickers were required to wear face scarves to prevent breathing in the adult insects. At the same time, few new pesticides were being registered for greenhouse crops. Chemical companies considered these to be ‘minor crops’, and registration cost could not be justified by the potential returns from such small markets. The frequent use and the need for high rates of application of chemicals in such closed environments caused growers to become very concerned about the effects on their own health and that of their workers. Not surprisingly, all these issues led to a renewed focus on biological control and mass rearing of natural enemies (beneficial predators and parasitoids). This was the birth of the commercial greenhouse biological control industry.

Development of a Commercial Biological Control Industry

Like many commercial ventures, the biological control industry initially consisted of a few small companies that worked closely with local government research facilities and agricultural advisory services. In Canada, *Encarsia* mass production began anew in Ontario in 1970–1972 at a government research centre (McClanahan, 1972). In 1973, scientists at Agriculture Canada formed a partnership with the local greenhouse industry, and parasitoid production costs were supported by the Ontario Greenhouse Vegetable Producers Marketing Board. Four million parasitoids were supplied to local growers. The next step was to commercially spin-off a private company. In 1974, Better Yield Insects Co. was formed in Ontario and it started the first commercial production of *E. formosa* and *Phytoseiulus persimilis*, a predatory mite for two-spotted spider mite. At the same time, mass rearing of *E. formosa* and *P. persimilis* began in western Canada. Thus, we were then producing BCAs for greenhouse growers in eastern and western Canada. The programme was so successful in western Canada that it led to the formation of a large-scale commercial biological control production facility, Applied Bio-Nomics Ltd. To ensure success of the production facility, a 5-year pilot programme, funded in part by Agriculture Canada and the British

Columbia Ministry of Agriculture, was set up to assist with production costs. We learned from our experience in Ontario that government has to assist financially in the establishment of such high-risk ventures. It was important to have the BCAs available when needed and in quantities sufficient to provide control. At this point we were just trying to develop a market place for these natural enemies. Soon after the initiation of this programme, the first large-scale releases of greenhouse BCAs were made, and during this 5-year period, Applied Bio-Nomics Ltd produced and distributed 6,195,000 predatory mites and 13,000,000 whitefly parasites, mostly to growers in British Columbia and Alberta. A survey in these two provinces in 1985 indicated that pesticide applications had been greatly reduced and biological control was being used by 85% of the cucumber growers and 38% of the tomato growers. In Ontario, 20% of the tomato area used *Encarsia* in the late 1970s, but by 1988, 75% were using biological control. The tide was changing rapidly from pesticides to biological control.

Development of an Integrated Pest Management Programme for Greenhouse Vegetables

The use of *E. formosa* for whitefly control and *P. persimilis* for spider mite control was the first step for greenhouse vegetable growers in switching to a biological control programme. For many reasons, the success rate with these natural enemies varied from region to region and within a growing season. For biological control to become the primary pest control strategy, it was apparent that other components of integrated pest management (IPM) needed to be developed for greenhouse crops. A critical component of IPM is early detection and monitoring of pest populations, in order to accurately time the implementation of control measures. We tested various sampling methods and found that the use of sticky yellow traps provided the best tool for early detection and monitoring for whiteflies (Gillespie and Quiring, 1987). This colour attracted a wide range of greenhouse pests and thus served as a useful sampling method for many greenhouse pests. When you develop a good idea, growers are very quick to implement the new technology. The provincial government in British Columbia sent ten free sticky yellow traps to all commercial growers to place in their greenhouses for the detection of whiteflies. Within a week of placing the traps in their greenhouses, growers were reporting populations of previously undetected whitefly and thrips. We were then on the road to developing a scouting programme for pests in greenhouse vegetables. However, we learned that placing a sticky card just anywhere in the crop did not produce the same results. The difference between a successful and unsuccessful IPM programme required that the traps be placed just above the top of the crop canopy for the most accurate estimate of pest densities. Growers quickly became accustomed to monitoring their pest densities and we turned our attention to other sampling methods and toward determining when economic injury levels occurred with pests such as the

western flower thrip, which became a major pest in the late 1980s and early 1990s.

In Alberta, a presence-absence sampling plan on sweet pepper was developed for western flower thrips and the predatory mite, *Neoseiulus cucumeris* (Steiner, 1990). This work confirmed that *N. cucumeris* was an effective BCA for thrips and that biological control provided a 20% yield increase in peppers compared to weekly applications of pesticides. This study was important because it provided clear values as to the economic benefit of using BCAs. A major challenge to implementation of biological control is the perception that biological control agents are much more expensive than pesticides. Generally, natural enemies can cost more than pesticides, but this can be offset by the benefits seen from an increase in fruit yield and quality, a decrease in health risks to the growers and a reduction in potential non-target effects in the environment. Today most greenhouse growers monitor pest densities on their crops either themselves or by using commercially available services.

Having developed sampling tools and plans for greenhouse pests and BCAs for two of the most important pests, the story does not stop here. There are many more pests in the greenhouse ecosystems. New invasive pests have occurred in greenhouse crops at a rate of approximately one new species per year over the past 20 years. These pests may be exotic insects, mites from warmer southerly locations or 'local' pests that invaded the greenhouse system from outdoor crops. For each new pest, new biological control alternatives along with IPM strategies need to be found. As mentioned earlier, western flower thrips became a serious pest of cucumber in British Columbia in 1984/5 and subsequently in cucumber and pepper crops worldwide. This pest had a profound worldwide impact on the IPM of greenhouse crops, because the only effective management strategy was chemical control as no BCAs were commercially available. This situation eventually resulted in a case of pesticide contamination of a cucumber crop in British Columbia where a number of consumers became sick (Hirsch *et al.*, 1987).

Shortly afterwards, we identified *N. cucumeris* as an effective control agent of western flower thrips on cucumbers and developed mass-rearing technology for it. Now BCAs were available for three major pests. During this period, however, minor pests, such as aphids, were controlled by broad-spectrum pesticides, which could no longer be used following the successful implementation of biological control for whiteflies, thrips and spider mites. In response to this dynamically changing pest community, mass-rearing and release methods were developed for the predatory aphid midge, *Aphidoletes aphidimyza*, and the aphid parasitoid, *Aphidius matricariae*. By 1990, an IPM programme was available for biological control of aphids in peppers and tomatoes using *A. aphidimyza* and *A. matricariae* (Gilkeson, 1990). In addition, the first mating-disruption programme was developed for tomato pinworm, a major pest of greenhouse systems in Ontario, (Wang *et al.*, 1997). New BCAs (predatory mites and bugs and parasitoids) continued to be developed on an as-needed basis during the 1990s.

Instead of always developing new BCAs for every pest/crop situation, research programmes were established to expand the target pest range of some generalist predators and the crops for which they could be used. As an example,

N. cucumeris was found to be effective for control of western flower thrips on tomato, contrary to the mindset that these mites would get stuck on the plants' sticky trichomes. *Dicyphus* was also found to be an effective BCA for thrips on tomatoes. The use of banker plants as reservoirs for parasitoids of aphids was first demonstrated in Europe, but was rapidly taken up by the industry in Canada. In the late 1990s, development of mullein plants as banker plants for establishment and retention of *Dicyphus* led to excellent control of whiteflies in greenhouse tomato crops (Sanchez *et al.*, 2003). This practice has been adopted in several regions of Canada (see Gillespie *et al.*, Chapter 14 this volume).

The arrival of microbials

The arrival of microbial BCAs proved to be another key tool in the toolbox of integrated control options for greenhouse pests. During the 1980s and 1990s, we demonstrated to growers that the bacterium *Bacillus thuringiensis* var. *israelensis* was highly effective for control of the larval stages of fungus gnats and *Bacillus thuringiensis* var. *kurstaki* for lepidopteran larvae, such as cabbage looper. *B. thuringiensis* is probably the most successful example of a microbial BCA being adopted for pest control, whether inside or outside the greenhouse (see Côté, Chapter 18 this volume). The growers quickly adopted these new BCAs, especially for control of lepidopteran pests. Another product that was rapidly adopted by growers was the use of entomopathogenic nematodes (see Ehlers, Chapter 15 this volume). *Steinernema carpocapsae* was originally advocated for control of fungus gnats in the early 1990s, but by the mid-1990s, *Steinernema feltiae* was the predominant species being promoted and used. There has been considerable interest in also adopting fungal BCAs, such as *Beauveria bassiana* and *Lecanicillium* (= *Verticillium*) *lecanii*, for pest control, dating back to 1985/6, when Applied Bio-Nomics Ltd conducted a full evaluation of *L. lecanii* as a BCA for greenhouse whitefly and western flower thrips. These products are registered in other countries but not in Canada. Efficacy trials with *B. bassiana* under Canadian conditions have shown that this product is effective for whitefly, thrips and aphid control. Non-target trials against greenhouse beneficials have also been completed (Shipp *et al.*, 2003) and an application for registration for *B. bassiana* has now been submitted in Canada.

Technology is not always rapidly adopted

The demonstration that a BCA is effective does not necessarily mean that growers will quickly have access to the product. Microbials are treated like pesticides under the regulations governing their registration. This process requires that a lot of hurdles be overcome, which are expensive and time consuming. Efforts to expedite the registration process led Agriculture and Agri-Food Canada to establish the Pest Management Centre, to work more closely with Health Canada to improve the time line and the efficiency of registrations of microbial BCAs. This will be more important in the future as there is a renewed interest in

microbial control agents with the many advances coming from biotechnology. Some of the new technologies that we are evaluating include the use of nucleopolyhedroviruses for cabbage looper control and the application of an atypical strain of *L. lecanii* (DAOM 198499) that is biologically active against both arthropods and fungi under greenhouse conditions (Askary *et al.*, 1998).

The rapid increase in the number of new commercially available BCAs (30 species) has resulted in a complex community of BCAs being released against a range of pest species. Although the development of new BCAs generally takes in excess of 10 years, in Canada, new BCAs are often commercially available in 1–5 years. A number of factors contribute to this rapid response. First, greenhouse crops are an international ecosystem, and research results are available usually in a timely fashion from Europe, Asia and elsewhere. This information is rapidly shared among researchers, extension advisors and growers. As a result, even if specific BCAs have not been identified for a particular pest problem, clues to potential successful agents are available. Secondly, unlike other consumers of BCAs, the greenhouse industry is a significant financial partner in the development of new agents. This partnership has often resulted in a tremendous demand for new agents, and commercial use may start in parallel with evaluation trials. Thirdly, the development, production and sale of, in particular, new arthropod BCAs have been relatively unfettered by regulatory constraints. The introduction of new agents from overseas has been considered the responsibility of the Canadian Food Inspection Agency under the Plant Protection Act, in collaboration with the North American Plant Protection Organization. In particular, the development and release of endemic North American natural enemies into greenhouses have not been a regulatory concern. In summary, extensive networking within North America and worldwide and excellent partnership with the greenhouse industry and the producers of BCAs has resulted in the development of an effective IPM programme in Canada.

Evolution of an Integrated Crop Management Approach

As our IPM approach evolved toward successful adoption by Canadian growers, we realized that biological control and IPM are really part of a larger integrated crop management approach. As reliance on broad-spectrum pesticides decreased and application of biological control increased, growers started to use bumblebees for pollination of greenhouse tomato. The use of bees resulted in better quality of fruit and significant higher yields (Kevan *et al.*, 1991). From the first commercial production of bees in 1989 in Ontario, tomato growers in Canada quickly adopted bumblebees for pollination by the early 1990s. We have also shown that bumblebees are efficient pollinators for sweet pepper, and today pepper growers introduce bees at the beginning and end of the crop to improve fruit quality and fruit set. The switch to bees from mechanical buzz pollination not only provided huge savings in labour but also provided a major impetus for using biological control. Bees are very sensitive to pesticides and, as a result, growers were willing to spend more money and effort in implementing biological-based IPM programmes. Thus, biological control not only displaced pesticides by virtue

of its equivalent efficacy for pest control, but it also provided a very significant improvement in yield and quality of produce by allowing the incorporation of bees into the production system. From a cost-benefit perspective biological control was an easy decision. In addition, pollinators are now being integrated as possible vectors of microbial control agents (see Kevan *et al.*, Chapter 35 this volume).

The greenhouse vegetable industry in Canada, and in the northern temperate climate countries, is a very technologically advanced industry. Computerized environmental control and fertigation systems are used by all growers. In Canada, most growers use portable personal data acquisition systems (e.g. Palm PDA) to monitor labour efficiency, pest 'hotspots', and track yield statistics in real time. Crops are grown in soilless media (e.g. rockwool, sawdust, coco fibre) or in nutrient film (i.e. only fertilizer solution). The size of greenhouse structures has increased to an average of 2.5 ha, but many exceed 4–8 ha and have a floor to gutter height approaching 6.4 m. Tomatoes are commonly grown using raised troughs (90–110 cm above the ground) to permit interplanting and to facilitate crop manipulation. Such changes have had significant impacts on production practices. The larger greenhouses and high gutters provide a much more stable greenhouse environment in the canopy and above it than was possible in the early structures. Computerized climate and fertigation systems and soilless growing media permit precise control of growing conditions.

Like many technological advances, though, a positive change in one area can create a challenge in another. Even with all the advantages of the sophisticated production system, we soon found that the new management practices were having significant impacts on pest and natural-enemy populations and communities that we did not foresee or understand. For example, we recommended that growers de-leaf tomatoes and cucumbers to increase air circulation and light penetration in the lower canopy and thereby provide an environment that excludes many plant diseases. However, if a grower is using *E. formosa* for whitefly control, removal of leaves bearing parasitized whitefly pupae will prevent any increases in the populations of this parasitoid. On the positive side, the larger greenhouses with high gutter heights create a more stable greenhouse climate, which in turn has reduced foliar disease incidence and resulted in better dispersal and reproduction of some BCAs.

One of the key constraints to the application of BCAs in greenhouses in Canada and in other northern temperate greenhouses is the effect of daylength on overwintering responses (diapause) in the BCA. Natural-enemy species from northern temperate climates tend to enter diapause in response to short daylengths in the spring, and decreasing daylength in the autumn, with the result that establishment (in spring) or control of pests (in autumn) fails. We developed solutions to this constraint through the artificial selection of non-diapausing strains of the BCA (Gilkeson and Hill, 1986) and by importing populations of predators from southern temperate regions (Gillespie and Quiring, 1993). Recently, the greenhouse industry has been exploring the economic potential for mid-winter production under supplemental lighting. This should alleviate the diapause problems, but it could also create significant carry-over of pests through the winter months.

In the mid-1990s, studies were initiated to see if we could improve the efficacy of BCAs in greenhouses by manipulating temperature, humidity, light intensity

and photoperiod. Greenhouses are enclosed production systems, and it is easy to accurately monitor and manipulate specific climatic conditions. First we developed climate models to predict conditions within the crop canopy and at the plant surface (Zhang *et al.*, 2002). These models were interfaced with the climate control systems to optimize energy efficiency, but also to modify the environment such that it led to an improvement in the efficacy of BCAs and other IPM strategies. The results of this research have allowed us to optimize the distance that introduced BCAs will disperse and to select the ideal conditions of humidity, temperature and light for predation and parasitism by predatory mites and parasitoids. Interestingly, we found that high temperatures and low humidity were by themselves an effective control measure at crop clean-up. Given the highly dynamic nature of the greenhouse environment and crop production technologies, we formed a team of researchers, extension advisors and greenhouse growers to develop a computer-based decision-support system 'Harrow Greenhouse Manager' for tomatoes and cucumbers to assist growers in making cost-effective crop management decisions (Clarke *et al.*, 1994).

Future for Biological Control of Greenhouse Crops in Canada

So what have we learned? We know that it was possible to switch greenhouse production from a reliance on pesticides to a reliance on biological control, such that today biological control is used on 85% of the greenhouse vegetable (tomato, cucumber and pepper) areas. Challenges and hurdles continually arise and can affect the continued successful development of biological control in Canada. Although most insect and mite pests have a biological control solution, increasing costs are forcing growers to minimize expenses and often at the expense of investment in BCAs. New strains and populations of aphids, spider mites and silverleaf whitefly are arising that are not as amenable to biological control as their predecessors. Furthermore, as a result of the ever increasing number of pests, we are releasing a much greater number of BCAs today than we did 10 years ago. This has resulted in a large increase in the number of BCA/pest interactions and has made biological control systems very complex. When pest control is reliant on a large number of BCA species, the probability that any one of them may fail increases with the greater diversity of agents used. When this happens, the only strategy available is to apply pesticides, and this disrupts the actions of all the other BCAs. Thus, there is a need to determine the optimum number of species of BCAs that should be introduced into a greenhouse system and their intraguild relationships.

Biological control over the last 80 years has become the main control strategy for greenhouse vegetables. Canada remains a world leader in the development of new BCAs and technologies to optimize this strategy. We have learned that development of an IPM programme for greenhouse vegetables is a dynamic, constantly evolving challenge. For successful implementation, one requires a team approach, a good understanding of the biology and ecology of the pests and their natural enemies, and flexibility as to which components of IPM are used, as these may be the same for each greenhouse operation but their deployment

may vary from region to region or time of the year. Little details can make the difference between success and failure, e.g. applying the BCAs as soon as you receive them. Perseverance is critical as success is often slow at the beginning, especially if pesticide residues are present in the greenhouse. Finally, one must always keep in mind the benefits derived from such non-chemical control measures, which include improved fruit quality and yield, without the concern over damaging the environment or health concerns due to exposure to pesticides.

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14

An Endemic Omnivorous Predator for Control of Greenhouse Pests

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Overview: Generalist natural enemies can be key members of biological control programmes. We believe that importation of generalist natural enemies for biological control should be avoided, and that endemic natural enemies should be used instead. We summarize our progress developing a generalist mirid, *Dicyphus hesperus*, for biological control in greenhouse tomato crops. Our success in locating a generalist mirid which can fill a niche in protected culture illustrates the potential for such approaches. This predator satisfies four of five preconditions that we set when we started the project and could potentially be used successfully as part of biological control programmes in greenhouses in North America.

Importance of Generalist Predators

In recent years, regulators have become increasingly reluctant to permit the introduction of new generalist natural enemies for biological control of pests to North America because of perceived risks of non-target impacts associated with

these species (Wajnberg *et al.*, 2001). None the less, generalist natural enemies are important members of natural-enemy communities that are managed for biological control of arthropod pests (Symondson *et al.*, 2002). In field-based agriculture, conservation biological control methods can be used to ensure that generalist natural-enemy communities are present to attack pest arthropod populations (Barbosa, 1998; Gurr *et al.*, 2004). Generalist predators may contribute to biological control of greenhouse pests but will have to be introduced into greenhouses deliberately, since invasions of predators from outside rarely occur in sufficient numbers. Because of the regulatory concerns mentioned above, any generalist predators used in greenhouses should be restricted to endemic species. In this context, exploration and evaluation of native fauna as potential sources of generalist biological control agents are extremely important. Below, we discuss the selection and development of one such natural enemy, *Dicyphus hesperus* (Hemiptera: Miridae), for biological control in greenhouses.

The Need for an Indigenous Generalist Predator

Like most complex stories, that of the discovery and development of *D. hesperus* has its beginnings in another time and place. In Europe, the generalist predator *Macrolophus caliginosus* Wagner (Hemiptera: Miridae) was found to be effective as a component of biological control programmes in greenhouse vegetable crops (Avilla *et al.*, 2004). As an omnivore, *M. caliginosus* feeds on both plants and prey, and can survive for short periods on tomato crops in the absence of prey (Malausa and Trottin-Caudal, 1996). Because *M. caliginosus* is a generalist that preys on a number of pest species present in greenhouses, it can play a role in the management of multiple pest species and allows reduced introductions of other, more expensive, specialized natural enemies. Most importantly, *M. caliginosus* is able to move freely on tomato crops, whereas most other cursorial predators are trapped on the glandular hairs on the crop (Fig. 14.1). The specialized tarsi of Dicyphini allow them to move freely on the surface of plants with glandular trichomes (Schuh and Slater, 1995).

The success of *M. caliginosus* in Europe prompted greenhouse tomato growers in North America, particularly in British Columbia, Canada, to lobby for its importation. Our consultation with Canadian regulatory authorities confirmed that permits for importation of *M. caliginosus* were unlikely to be issued in either Canada or the USA. Therefore, a project to develop a native natural-enemy species, with the characteristics of *M. caliginosus*, was initiated. This project was a collaborative partnership with stakeholders that had a vested interest in a successful outcome. Agriculture and Agri-Food Canada, the British Columbia Greenhouse Research Council, BC Investment Agriculture Foundation, Koppert Canada, Applied Bionomics Ltd, BioBest Canada, and the NSERC Biocontrol Network all contributed major funding.

Exploration and Screening

We felt that suitable candidates from North America would probably occur in the same family and subfamily as *M. caliginosus* because many members of this

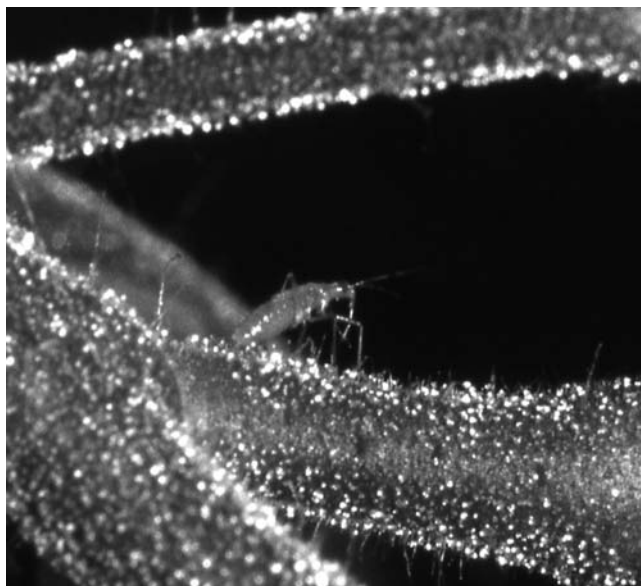


Fig. 14.1. Nymphs of *Dicyphus hesperus* have specially adapted tarsi that allow them to walk easily over sticky glandular hairs that can entrap other insects.

group share similar biological characteristics. We developed a protocol for screening omnivorous predatory Miridae that used several *a priori* criteria: (i) all stages had to be able to live on tomato plants; (ii) the species had to be relatively widespread in North America; (iii) the species could not be a known plant pest; (iv) it had to complete development on greenhouse whitefly and two-spotted spider mite; (v) it should have a relatively short development time and high fecundity; and (vi) it should complete development and increase in numbers on tomato plants in the absence of prey. A relatively large number of species in the family Miridae, subfamily Bryocorninae, were screened as potential agents (Table 14.1). The major failing of the survey was that we were constrained to western North America for logistical reasons. Most of the species we screened failed in more than one criterion.

D. hesperus was chosen as the candidate for further exploration, based on its meeting four of our five criteria. We found that *D. hesperus* does not complete development or lay eggs on tomato plants in the absence of prey (McGregor *et al.*, 1999). It is, however, relatively widely distributed (Cassis, 1984), and completes development on a diet of either spider mites or whitefly on tomato plants (McGregor *et al.*, 1999). Most importantly, all stages can move freely on all parts of tomato plants, including those parts with extensive glandular hairs. The life cycle was found to be relatively short at temperatures typical of greenhouse vegetable culture (Gillespie *et al.*, 2004), and there were no records of this species causing damage to agricultural crops.

The original test population was collected at 49° 36' N 119° 40' W, elevation 334 m, on catnip, *Nepeta cataria* (Lamiaceae), mullein, *Verbascum thapsus* (Scrophulariaceae) and other plants, at Summerland, British Columbia, Canada. In preliminary work, we found that adults of this population entered reproductive diapause at a daylength of 15 h. Supplementary lighting was not used in

Table 14.1. Species of Miridae, subfamily Bryocorinae, screened as potential generalist biological control agents. All species were field collected in south-western British Columbia.

Species	Host plants	Preliminary screening results
<i>Dicyphus fasciolus</i>	Weedy vegetation	Rare, unable to assess
<i>Dicyphus discrepans</i>	<i>Geranium</i> spp.	Predatory, but much longer life cycle than <i>D. hesperus</i>
<i>Dicyphus hesperus</i>	Scrophulariaceae, Solanaceae, etc.	
<i>Dicyphus pallidicornis</i>	<i>Digitalis purpurea</i>	Monophagous on <i>D. purpurea</i>
<i>Campyloneura virgula</i>	Deciduous trees?	Apparently univoltine? Did not reproduce on tomato plants
<i>Macrolophus rivalis</i>	<i>Ribes</i> spp., <i>Rubus</i> spp.	Rare, unable to assess From subalpine forests
<i>Tupiocoris rubi</i>	<i>Rosa</i> spp.	Herbivorous
<i>Tupiocoris</i> sp.	<i>Grindelia integrifolia</i>	Probably herbivorous
<i>Usingerella bakeri</i>	<i>Ribes</i> spp.	Long life cycle, from subalpine forests

greenhouse vegetable production at the time this screening was done, so this diapause trigger would have limited the use of this predator to the summer months in much of Canada. This highlights the need to explore widely for potential natural enemies. Taxonomic and systematics expertise are necessary for this kind of activity, and a phylogenetic perspective is essential. In the course of exploring in the Sierra Nevada mountains of California, a population of *D. hesperus* was collected at 35° 43' N, 116° 49' W, elevation 300 m on white-stem hedgenettle, *Stachys albens* Gray (Lamiaceae) near Woody, California, USA. Its critical daylength was determined to be around 13.5 h, and its responses to daylength were facultative and highly modified by temperature (Gillespie and Quiring, 2005). Therefore, the potential of this population as a biological control for arthropod pests on greenhouse-grown tomato plants was further evaluated.

Efficacy

We found that releases of *D. hesperus* on tomato crops in research greenhouses provided control of whitefly and spider mites, although pest outbreaks generally occurred before populations were brought under control. A combination of a relatively long oviposition period, and an inability to reproduce or develop on tomato plants in the absence of prey produced a delay in the response of *D. hesperus* populations to prey population increases. Moreover, populations did not persist in the greenhouse after pest populations had been reduced.

Most growers who tried using *D. hesperus* soon after its discovery also found that it failed to persist in greenhouses, and that it took a very long time to increase to numbers that were effective against pest populations. This was especially true for growers who introduced *D. hesperus* when prey populations were very low or absent. Therefore, we explored alternative methods for introducing *D. hesperus* to greenhouses. Based on host-plant-preferences research, we found that mullein, *Verbascum thapsus* L., was a highly preferred plant host that allowed reproduction and development in the absence of prey (Sanchez *et al.*, 2004). Introductions of *D. hesperus* on mullein plants placed within the tomato crop enhanced the establishment of *D. hesperus* and increased its impact on pest populations (Sanchez *et al.*, 2003). Adults of *D. hesperus* move relatively freely between mullein plants placed on the floor of greenhouses and tomato plants grown on trellises above, and placement of mullein plants in hanging baskets within the tomato plant canopy has been shown to facilitate the movement of *D. hesperus* on to the crop. This is the first time that an increase in crop diversity via the introduction of alternative host or insectary plants (mullein) has been proven to contribute to the response of a predatory mirid and subsequent pest control in greenhouses. In this case, movement of *D. hesperus* between crop plants and mullein results in an earlier decline in the pest population. Growers who have tried this approach in greenhouses have found it to be successful.

Introductions of *D. hesperus* have also been shown to reduce numbers of other common greenhouse pests, such as western flower thrips, which can be reduced below economically damaging levels on greenhouse tomato crops. Introductions of *D. hesperus* on gerbera daisies (*Gerbera jamesonii*) provided excellent control of greenhouse whitefly, thrips, leaf miners and two-spotted spider mites. In addition, releases of *D. hesperus* in commercial pepper crops provided some reduction of aphid populations (Luczynski *et al.*, 2002) and may have contributed to reductions in numbers of thrips. This predator is reported to be highly successful as a biological control for a variety of pests on aubergines.

Issues and Constraints

D. hesperus is an omnivorous insect. It requires both plant and prey food sources to complete development (Gillespie and McGregor, 2000). In the presence of high-quality prey, *D. hesperus* is able to complete development on most plant species, but without prey, development of nymphs only occurs on mullein and, to a very limited extent, on pepper and catnip (Sanchez *et al.*, 2004). Plant species affects recruitment and retention of adults into patches, and prey species also affects retention in patches. Adults of *D. hesperus* tend to remain on mullein much longer than on pepper and tomato plants, which allows retention of predators in crops after prey have been reduced in numbers.

The most controversial aspect to the use of *D. hesperus* as a biological control agent is the damage caused to tomato fruits by adults and nymphs feeding on fruit. McGregor *et al.* (2000) showed that in the presence of leaf material or prey in small arenas, *D. hesperus* did not feed to a great extent on ripe tomato fruits; however, *D. hesperus* tend not to feed on ripe fruit. Blemishing is primarily

caused by feeding on young, green fruit, even though blemishes are only apparent on ripe fruit at harvest. Provision of alternative, non-prey foods can dramatically reduce feeding on green fruits. Blemishing by *D. hesperus* on tomato is generally not serious enough to cause fruits to be reduced in grade, so use of the predator on tomato crops is not constrained by blemishing. On gerbera crops, however, adult and immature stages of *D. hesperus* feed on the centre of developing flowers, which may cause deformation of the blossom and downgrading of the flower. The use of *D. hesperus* on gerbera may be constrained by the feeding damage. It does not appear that *D. hesperus* feeds on pepper or aubergine fruits to a noticeable extent.

Success of the Project

Although research and development continues on *D. hesperus*, it is possible to make some preliminary remarks about the success of the research project that resulted in its development as a biological control agent. At present, *D. hesperus* is used in greenhouse tomato crops in Quebec, Ontario and British Columbia. It appears to be particularly useful when tomato crops are grown under lights in winter. Where growers interplant new plants among mature plants, *D. hesperus* moves readily to the new plants and provides control of pests moving from the old plants. Growers have found it slow to increase in numbers in response to pest invasions, although once established it provides adequate to excellent control of whitefly and may also aid in control of thrips on tomato crops. One of the target pests for the original project was two-spotted spider mites on tomato crops. There are some anecdotal accounts of biocontrol of spider mites by *D. hesperus*, but it has not generally been seen to contribute substantially to control of spider mites in commercial settings.

Intra-guild predation may be a consideration in the application of *D. hesperus* in greenhouses where other natural enemies are present. Thus far, it appears that *D. hesperus* preys on other natural enemies in proportion to their abundance. In the long run, this may have a stabilizing effect on prey populations by preventing extinction of the prey that both *D. hesperus* and the specialist natural enemies depend on for survival. This has yet to be fully investigated.

Establishment of *D. hesperus* in the absence of pests has proved difficult, and although the addition of mullein into crops has alleviated this situation somewhat, the practice is expensive. Moreover, the cost of production of *D. hesperus* is high, due partly to the long life cycle, but mainly to the cost of *Ephestia kuehniella* eggs, which are used as a food source. Reducing the cost of production would have a salutary effect, and would allow growers to introduce large numbers in response to the presence of pests.

Future Research

Biological control of pests on greenhouse crops remains a challenge. Fuel and labour costs increasingly constrain the profitability of greenhouse farms.

Growers may elect to reduce their use of biological control products in order to reduce costs and, under economic constraints, may be more likely to opt for the short-term risks involved in the use of chemical pesticides. Crop management practices are continuously changing, and these changes impact on the efficacy of the biological control agents. Economic injury thresholds are continuously decreasing due to a low tolerance for insect residues such as honeydew or frass. Invasions of pests for which there are no biological controls may dictate that pesticides must be applied, which then impacts on existing natural enemy communities. The short generation times for greenhouse pests results in a rapid selection for resistance to insecticides, and the loss of essential IPM tools.

The active use of generalist natural enemies as part of a biological control programme in greenhouse crops could alleviate many of these problems. The use of generalist natural enemies as part of an IPM programme can reduce the frequency of pesticide applications and can both reduce the speed with which resistance evolves and lessen its impact when it does. Moreover, generalist natural enemies in greenhouses can provide a first line of defence against pest invasions. Finally, if tolerances for residues dictate that pest populations must be lower than can be maintained with specialist natural enemies alone, then the addition of generalist biological control agents to the natural-enemy community may assist in achieving those population targets.

A greater understanding of the biology and ecology of generalist natural enemies is required in order to use them effectively. The concept of a generalist natural enemy, i.e. a natural enemy that exercises no discrimination, does not reflect the biology or ecology of these species. Generalist natural enemies are highly sensitive to physical environments, plant substrates and communities, prey quality and abundance, and the presence of competitors. Our understanding of these and other elements of the biology and ecology is pivotal to the successful application of generalist natural enemies in biological control.

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15 Entomopathogenic Nematodes: from Science to Commercial Use

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Overview: This chapter describes the development and commercialization of entomopathogenic nematodes (EPN) as biological control agents. The research and development was always accompanied by alliances with companies active in plant protection and thus the strategies of the different commercial enterprises are also described. As much of this history is part of my own odyssey through the world of biological control, this chapter also describes part of my scientific and commercial career.

Entomopathogenic Nematodes

Entomopathogenic nematodes (EPNs) were first used in the biological control of Japanese beetle, *Popillia japonica*, grubs in the USA (Glaser and Farrell, 1935). They are small worms of the genera *Steinernema* and *Heterorhabditis* living in a close symbiotic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, which are used in biological control of insects in cryptic environments. Their use is summarized in Grewal *et al.* (2005) and their biology in Gaugler (2002).

The free-living stage within the life cycle of EPN is the morphologically distinct infective dauer juvenile (DJ), which is formed as a response to depleting food sources and adverse environmental conditions. The DJ is a free-living third juvenile stage, which is well adapted to long-term survival in the soil. It does not feed and can survive periods of starvation for over a year owing to its lipid reserves. The DJs search for hosts and invade them through natural openings (mouth, anus, tracheae) or also directly through the cuticle. In the haemocoel they leave the developmentally arrested DJ stage, a step called 'recovery'. The recovery-inducing signal is called 'food signal'. During recovery, the DJs release the symbiont bacterial cells into the haemocoel of the host insect. The bacteria produce toxins and other metabolites, which contribute to overcoming the insect's defence mechanisms and to killing the insect within approximately 2 days after nematode invasion.

(Ciche *et al.*, 2006). After insect death, the bacteria proliferate and produce suitable conditions for nematode reproduction. The nematodes feed on the symbiont cells, develop to adults and produce offspring. As long as abundant nutrients are available, additional adult generations will develop. When the nutrients are consumed, the offspring develop to pre-dauer second-stage juveniles (J2d), which retain the symbiotic bacteria in the intestine and develop to DJ, which leave the insect cadaver in search of other hosts (Fig. 15.1).

Learning About Nematodes the Hard Way

During the 1980s, the city of Hamburg expanded its water supply area into the south-west of Schleswig-Holstein state, a region of intensive production of nursery stock. The region became a water protection area, and governments wanted to limit the use of soil insecticides to prevent groundwater contamination. The major pest problem of yew, rhododendron and many other nursery plants is the black vine weevil (BVW), *Otiorhynchus sulcatus*. Publications indicated that EPNs had potential to control larvae of the BVW, so the idea was born to further evaluate this possibility. Preliminary tests during my diploma thesis at the Christian-Albrechts-University (CAU) in Kiel, under Professor Urs Wyss, revealed a higher control potential with *Steinernema glaseri* than with *Steinernema carpocapsae*.

I received the first EPN material from Richard Sikora (Univ. Bonn, Germany), and as the insect host required for propagation (*Galleria mellonella*) was not available in Kiel. I immediately tried propagation *in vitro* on dog food agar and later on three-dimensional sponge medium inoculated with surface-sterilized infective dauer juveniles (DJ) according to Bedding (1981) and Wouts (1981). Ignorant of microbial techniques, I did not know how to culture and identify bacteria, so I just inoculated DJ from stock cultures kept in 0.1% formalin into autoclaved media in flasks. The first culture cycles were usually successful and enough material was available to perform pot trials against the BVW. The control results were

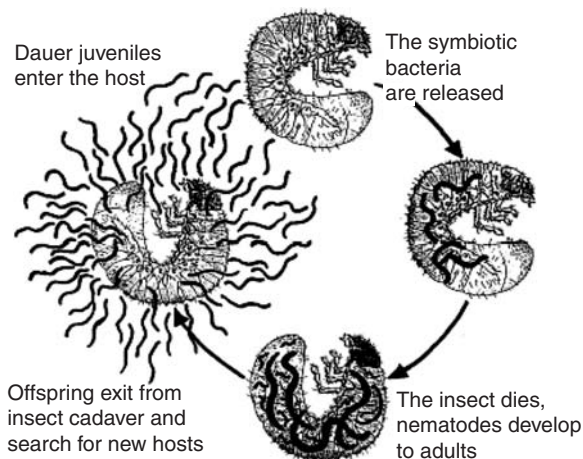


Fig. 15.1. Life cycle of entomopathogenic nematodes in a scarabaeid larva.

promising, but when I tried to produce more nematodes and used *in vitro* cultures as an inoculum, nematode propagation always ceased and I only produced stink. Was this a problem of contamination or phase variation of the symbiotic bacteria? Phase variation is a typical phenomenon of nematode symbiotic bacteria, which occurs in the primary phase in the nematode and in insects, but when cultured *in vitro*, the bacteria often switch to the secondary phase, which can be less effective for nematode reproduction. This provided a challenge so I decided to continue in the field of EPNs after my diploma thesis and studies.

The Idea for Mass Production in Liquid Culture is Born

If these fantastic nematodes were ever to be commercialized, methods for their economical mass production would be needed. Production on a larger-scale solid medium using plastic bags (Bedding, 1984) required considerable labour and handling. Would it be possible to produce EPN in liquid media using standard bioreactors used in the biotechnology industry?

The First Steps towards Liquid Culture Production

At the time, the only report on nematode culture in liquid was by Stoll (1952), who had grown *S. carpocapsae* in bacteria-free cultures without much success. I contacted Prof. Erko Stackebrandt at the Department for Microbiology, University of Kiel, and explained my problems with phase variation or possible contamination. Erko, an entrepreneur in bacterial phylogeny, had never heard of *Xenorhabdus* and was even more surprised when I told him that they were classified in the family Enterobacteriaceae. Within the next months I was working in his department on a 16S rRNA sequence catalogue, and results were published supporting the designation in the Enterobacteriaceae. At the same time, I had my first experience in microbiology, and we built a small laboratory equipped for *in vitro* culturing of EPNs.

A research proposal to the German Ministry for Science and Technology was rejected because we lacked industry co-financing. Professor Wyss succeeded in getting the chemical company BASF (Limburgerhof, Germany) interested in the project. BASF was screening microorganisms for metabolites with potential use in pharmacy or plant protection. Nematode endosymbionts were something not every competitor would be able to access.

We isolated nematodes and their symbiotic bacteria, and BASF found several interesting metabolites in the supernatant. During this 3-year project, we first learned how to distinguish between contaminants and phase variants and then investigated the influence of phase variation on nematode reproduction. We also learned how to avoid negative impacts of phase variation and developed a verifiable procedure to produce contamination-free nematode inoculum. Results of the project were summarized in my PhD thesis. At the same time, we successfully cultured *Heterorhabditis bacteriophora* in a liquid medium in shaker flasks, and half a year later, in a lab-scale 10-litre bioreactor. Although results were

highly variable, we applied for more bioreactors and tried to get our project prolonged.

The Lab-scale Project – Understanding Nematode Biology in Liquid Cultures

BASF had major difficulties with the stability of the bacterial metabolite production owing to phase variation, and so continuation of the project area seemed unattractive. Perhaps the wrong decision at that time, as today many patents have been filed on the metabolites of nematode symbiotic bacteria (Webster *et al.*, in Gaugler, 2002), including the TCA insecticidal protein with potential to make transgenic crops resistant against insect attack (Bowen *et al.*, 1998). Fortunately, another industry partner, a medium-sized enterprise, Neudorff (Emmerthal, Germany), stepped in. It had just invested in production of predatory midges (*Aphidoletes aphidimyza*) for biological control of aphids and also sold EPNs, which were produced on solid media by the Dutch company De Groene Vlieg (Nieuwe Tonge). This paved the way for continued German federal government financial support.

Bioenterprise, a subsidiary of Biotechnology Australia, patented a method for liquid culture of nematodes (Pace *et al.*, 1986), but did not commercially exploit it as they produced nematodes on solid media. At the same time, Biosys (Palo Alto, California, USA) developed liquid culture technology for the nematode *S. carpocapsae*. I discussed cooperation between them and Neudorff to develop liquid culture of *Heterorhabditis megidis*; however, they were not interested as they considered their company was far ahead in the development of liquid culture. They had just started commercial production in liquid media of 10,000-litre batches of *S. carpocapsae*, *Steinernema feltiae* and *Steinernema riobrave*, but in the end, they never managed to produce heterorhabditids commercially in liquid culture (Georgis, in Gaugler, 2002).

In October 1990, we moved from Kiel University into a new 300 m² laboratory in Raisdorf, at an old whisky factory. Two 10-litre and three 5-litre lab-scale bioreactors were installed and tested using flask cultures, and we were surprised at our success. I was working with a team of PhD students: Karl Hermann Osterfeld, studying effects of process conditions on nematode yields, his wife Karina, on bacterial phase shift, and Stefan Lunau, on nematode development and DJ recovery. Yields obtained with a German isolate of *H. megidis* were highly variable (between 21,000 and 68,000 DJs/ml) and the process time varied from 13 to 25 days. We identified the variable development of the nematode inoculum as the major reason for process failures. Nematode inoculation was by transferring suspensions of mainly DJs from prior liquid flask cultures. Instead of developing to fourth-stage juveniles and further to hermaphrodites, the DJs often remained in the dauer stage. Recovery was variable, resulting in non-synchronous development, lower yields and prolonged process time. We had at least identified the major problems.

Table 15.1. Influence of low or high DJ recovery on the population development and DJ yield

Low DJ recovery	High DJ recovery
low adult population density	high adult population density
good food supply	low food supply
high number of eggs laid	low number of eggs laid
long hermaphrodites	short hermaphrodites
late entrance into <i>endotokia matricida</i>	early entrance into <i>endotokia matricida</i>
high population of second-generation amphimictic stages ¹	low population of second-generation amphimictic stages ¹
DJs of hermaphrodites recover	no recovery of DJs of hermaphrodites
two generation process	one generation process
low yields	high yields
many DJs from laid eggs (low quality)	low number of DJs from laid eggs
DJs of first generation lose quality until harvest	DJs mainly from <i>endotokia matricida</i> ²

¹which have no offspring in liquid culture

²of excellent quality

of the DJ inoculum produced lower food conditions and the majority of the offspring developed into DJs, again without going through a second generation of adults. Thus the process yielded more DJs within a shorter time. Figure 15.3 sketches the life cycle of heterorhabditid nematodes and Table 15.1 describes the influence of high or low DJ recovery on the nematode development in liquid culture. Although still struggling with biological and technical problems, we had gathered enough experience and data to be able to plan the next project phase to increase process stability and test the protocol at the pilot-plant scale.

The Pilot-scale Project – with the Help of Venture Capital Investors

Neudorff continued support of our project as they experienced difficulties in obtaining nematodes from solid-state production on contract due to problems with stability in the solid-state process. With 3 years experience of liquid culture in lab-scale bioreactors, we planned an industrial-scale plant, which needed an investment of approximately €0.5 million. We considered that the bacterial pre-culture and the phase of dauer recovery would need precise management of the process conditions, which could only be provided by well-controlled bioreactors equipped with stirrer and control systems for oxygen, pH, pressure and temperature. The hypothesis was that, as the nematodes had developed to hermaphrodites, the basis for the yield was set, the fragile culture phase had passed, and cultures

could be transferred to less well-controlled and cheaper tanks. Harvesting of nematodes was supposed to be done from these tanks. One bioreactor would have served to fill 3–4 tanks. However, Neudorff did not have the capital for investment in such a production plant, but it was willing to support testing the technology at the pilot-plant scale. The local technology foundation Schleswig-Holstein (TSSH) was more positive about the progress we made and supported our proposal to purchase a 500-litre bioreactor.

I was happy to come in contact with Bruce Carlton, scientific director of the company Ecogen Inc. (Langhorne, Pennsylvania), during the annual meeting of the Society for Invertebrate Pathology in Heidelberg in 1992, who expressed interest in production technology for entomopathogenic nematodes. Ecogen, a venture capital enterprise, had developed and marketed several genetically improved strains of *Bacillus thuringiensis*. The company had bought Bioenterprise Ltd (Australia) from Hoechst (Frankfurt, Germany), which provided nematodes produced on solid media using plastic bags. Ecogen had attracted venture capital in the Nasdaq stock market and invested in four major areas: development of innovative formulation of *Bt*, post harvest and powdery mildew control, and EPNs. Projecting market sales of several million US\$, it was clear that these amounts could only be provided from large-scale liquid cultures. Ecogen offered to step into the ongoing project with Neudorff and agreed to provide an exclusive sales agreement for Germany and neighbouring countries. In return, Neudorff agreed to continue with one research scientist concentrating on quality control and development of new markets. Ecogen provided research funds for the CAU to continue R&D in recovery, phase variation and improvement of process control, and at the same time wanted to start a business and equip a research laboratory in the floor above the university facilities. They formed Ecogen Bio Germany and hired three post-docs (of which Krasomil-Osterfeld and Osterfeld had received their degrees during the prior project) and a technician to continue the commercial development of liquid culture. The 3-year research project at the CAU focused on fundamental aspects of nematode biology. The focus at Ecogen Bio was on scaling-up and technical improvement. This combination laid the basis for an industrial-scale production facility. At least, this was my vision. However, Ecogen had a commitment with the Italy-based company 3A in Todi, where they had started a joint-venture biocontrol laboratory, and the Italian partner expected a major technology transfer to Italy including know-how in EPN liquid culture production.

Ecogen also started cooperation with Sylvan Foods, which produced mushroom spawn, and the *in vitro* production facilities seemed to fit well for production of EPN on solid culture (Gaugler and Han, in Gaugler, 2002). Although much handling was still involved and problems with contamination remained, Ecogen considered this technology cheaper and more effective than production in liquid. At the time, Ecogen wanted us to switch research to *H. bacteriophora*. Within a year we reached yields of > 200,000 DJs/ml. After an appropriate symbiotic bacterium had been isolated, yields were doubled compared to the culture of *H. megidis* and process stability was significantly improved. The symbiont of the latter nematode was more frequently switching to the secondary phase, whereas we had difficulties in producing secondary-phase cultures of the *H. bacteriophora* symbiont. However, the problems with recovery of the DJs remained.

Major progress was made when we realized that a food signal triggering DJ recovery was excreted by the symbiotic bacterium *Photorhabdus luminescens*. After studying the food signal which induces DJ recovery, we realized that the DJs hardly develop without the bacterial pre-culture, as the artificial media components lack any kind of food signal inducing the exit from the DJ stage. In nature, the DJs enter an insect host and the food signal in the insect induces recovery in usually 100% of the DJs. With the bacterial food signal, the recovery varied between 10 and 80%. To understand the function of the bacterial food signal, one needs to consider when the signal is important during the life cycle of the nematode. It is absent in the haemocoel during DJ invasion. Its function is to promote the development of second-generation adults (high concentration) or induce the formation to DJs (low concentration). At a later stage, the bacterial food signal can induce recovery of DJs exiting the carcass of the hermaphrodite after passing through *endotokia matricida* (Fig. 15.3). Should the bacterial food signal trigger 100% of DJs to recover, the food supply would not be sufficient for all hermaphrodites to produce offspring. Overcrowding would be the result. Thus a lower percentage of recovery causes the development of fewer hermaphrodites that can still produce DJs with enough fat reserves. As EPN production in artificial media can only make use of the bacterial food signal as other signals are not yet identified, it was important to study process factors which can enhance recovery based on this food signal.

A high recovery of DJs is essential to synchronize nematode population development in liquid culture, to enhance process stability and to increase yields. We therefore investigated the influence of several process parameters on DJ recovery. Carbon dioxide concentration seemed to have a major impact. We discovered that adding CO₂ to the air input provided a higher and earlier recovery and increased the DJ yield from 60,000 to 160,000 DJs/ml (Ehlers, 2001). Another approach was to increase food-signal production of the nematodes by fed-batch conditions. By feeding nutrients (glucose) during the logarithmic growth phase of the symbiont, the bacterial dry mass was doubled and the percentage of cells with crystalline inclusion protein was increased (Jeffke *et al.*, 2000). However, nematode cultures were too variable to prove that fed-batch conditions also increased the recovery and the yields.

Certainly the results obtained in flasks and small-scale experiments helped us to understand the biology and the influence of process conditions; however, it was a tremendous advantage to scale up to 10 litres and further to the pilot-plant scale of 500 litres. The handling of the in-situ sterilized equipment, the technical stability of these machines compared to bench-top bioreactors, and the possibility of measuring process parameters and taking many samples without any influence on the process provided conditions for rapid progress. We were able to install the pilot plant at the laboratory of Ecogen Bio Germany, stabilize yields and reduce process time and stepped into downstream processing. EPNs were provided to other partners within the Ecogen team for research on quality, storage and formulation. Producing in 500-litre batches, we finally had enough nematodes of good quality to provide extensive field tests. Ecogen required us to send the nematodes to Todi, Italy for quality control. From there they travelled to Ecogen USA for formulating, and the formulated product was then returned to Italy for distribution to partners for field testing. This arrangement only produced

dead nematodes and made us understand that the direct transport of EPN material to the user is crucial for successful introduction of EPN into biocontrol practice.

Biotechnology stocks were under pressure. Ecogen's stocks crashed and the investment bank managing Ecogen's assets and the owner of more than two-thirds of its stocks went bankrupt. Ecogen decided to close its subsidiaries in Raisdorf and Todi. All available data and technology were transferred to 3 A in Italy. In return, 3 A provided \$1.2 million to Ecogen Bio Germany, which was transferred to the USA headquarters in an attempt to stabilize its financial situation. At Raisdorf, the equipment was transferred to the University in compensation for outstanding R&D funds.

This was the end of the engagement of venture capital into EPN development. Biosys had to declare bankruptcy as they had overestimated their sales and had signed up for ten 80,000-litre bioreactors, which they could not fill (Georgis, in Gaugler, 2002). Thermo Triology took over but soon sold the business to Certis USA.

The Industrial-scale Project – Going Commercial

After working with three different industry partners and finally being left with hardly anything in my hands, except for some pilot-plant equipment, I was fed up with public-private partnerships. Neudorff had at least given me a future perspective with an agreement on royalties related to the amount of sales. I submitted a proposal to the EU for support of the further development of liquid culture for EPN in an attempt to continue at least at the university level. This proposal was rejected because there was no industry partner involved.

Around this time, a school friend of my wife, Tillmann Frank, asked me whether I could drive his car back from Sweden. He was going to pick up the sailing yacht he had bought. I accepted and took this opportunity to meet with the representative of Bionema in Stockholm to provide him with my plans for a liquid-culture production facility. The company had built a small business based on the knowledge of solid-state production of Martin Burman from the University of Umea. However, we could not agree on conditions for my participation as M. Burman had doubts that liquid culture could yield high-quality nematodes, as previous field experiments with *H. bacteriophora* from liquid culture provided by Biosys were not promising (Georgis and Gaugler, 1991). During this trip I told Tillmann my story and a month later we started our own business, together with Arne Peters, who had worked at 3 A in Italy. We re-submitted the project proposal to the EU. In January 1997, I was informed that the EU proposal had a good chance of acceptance. The EU project Pronema started in September 1997 and provided financial support to continue the scientific studies on the life cycle and fundamental process parameters at the University of Kiel and at the same time adequately fund the company to push forward the development of commercial production and downstream processing including harvest, cleaning, storage, formulation and packaging. Within the Pronema project, Stefan Johnigk's research concentrated on the life cycle and population dynamics of

the nematodes. The fundamental analysis of the life cycle, in particular the description of the *endotokia matricida*, underlined the importance of the management of the nematode population dynamics. Best-quality DJs (accumulation of fat reserves) were achieved after they had developed inside the hermaphrodites (Fig. 15.3). The *endotokia matricida* is the adaptation for survival even when conditions deteriorate. The body of the mother provides the exact amount of food for the offspring in the uterus at the beginning of the *endotokia matricida*, and the DJs can grow without major influence from the surrounding environment in the insect cadaver or the bioreactor medium (Ehlers, 2001).

The results of several commercial production runs indicated that, with a given medium and resulting bacterial density, a specific hermaphrodite density will result in a maximum DJ yield. To reach this yield, the hermaphrodite density has to reach a minimum of 3000 DJ/ml in the medium we used. As such a hermaphrodite density could not be reached by inoculation of DJs owing to the variable recovery, we continued to study conditions that could promote recovery. We found that the maximum food-signal activity was during the entry into the stationary phase. We could also correlate the maximum activity of the food signal with a shift in the respiration coefficient and a drop of the pH in the medium. The results served to define the moment of nematode inoculation in order to maximize DJ recovery (Johnigk *et al.*, 2004).

The major source of variability, however, was the nematode inoculum itself. Spending years in research has not led to a complete understanding of the DJ recovery. However, we have learned how to handle cultures to secure yields and quality of EPNs of the genus *Heterorhabditis*. In order to manage population dynamics, the process needs to be inoculated at the optimal time. To obtain the necessary hermaphrodite density, one must know the percentage of DJs which will recover in the inoculum batch. This can be evaluated 5 days prior to inoculation and then the population dynamics of the nematodes are manageable.

Biotechnology, however, needs both an understanding of the biology and the use of available technology. After a few runs in the pilot plant, Arne was able to continuously improve the process. Within a short time, he developed standard operation protocols for production and downstream processing using a separator (Fig. 15.4), which concentrates the shear-resistant DJs and at the same time kills all remaining adults. A consecutive passage through a sifter (Fig. 15.5) cleans the suspension of remaining bacteria and other residues, and the DJs are then passed into cooled storage tanks (Fig. 15.6). In order to maintain high viability during transport, the DJs are mixed with clay and then packed in plastic bags (Fig. 15.7). Prior to marketing, each batch is checked in quality-control assays with *Tenebrio molitor* larvae.

Marketing and Market Confidence

Soon after we started marketing, it became obvious that we were unable to meet the demands of the market in the pilot plant. Additional bioreactors were needed, and we installed a 500-litre bubble column within the first year and

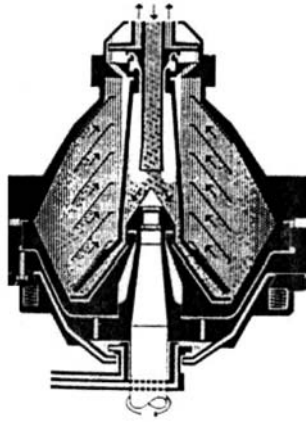


Fig. 15.4 Separator used to process the DJ suspension after production in the bioreactor. This centrifuge segregates nematodes from spent medium and bacteria.

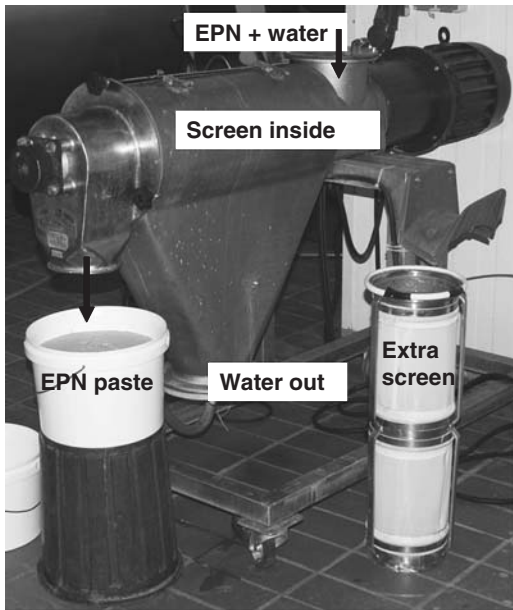


Fig. 15.5. Sifter used to run nematodes over a screen to wash out remaining bacteria and debris smaller than the nematodes and concentrate the DJ suspension. Rubber policemen are turning over the screen.

were fortunate to buy a used 3000-litre fermentor equipped with the same stirrer as we were using in the pilot plant. The internal loop produced by a marine impeller provides excellent oxygen transfer at low shear forces (Fig. 15.8). After re-installing the bioreactor in 1998, the critical moment was reached when we first inoculated this machine with EPN. Usually scaling up reduces the yields per volume. We were surprised to harvest even higher yields than in the pilot plant, surpassing 200,000 DJ/ml. This made us review the design of the pilot plant and adjust the internal loop system, resulting in equally high yields as in the 500-litre tank.

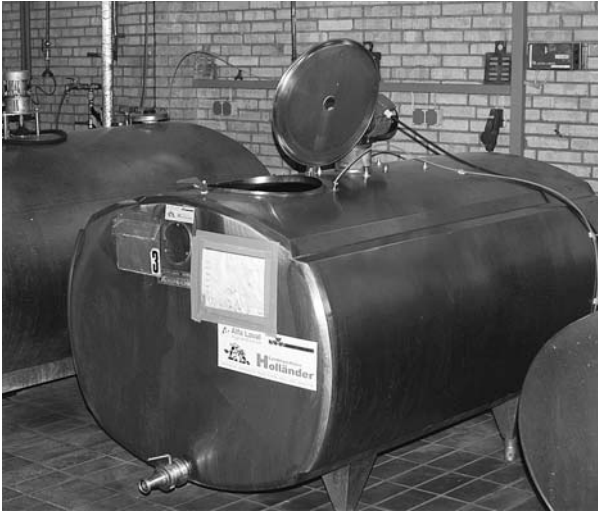


Fig. 15.6. Cooling tanks.

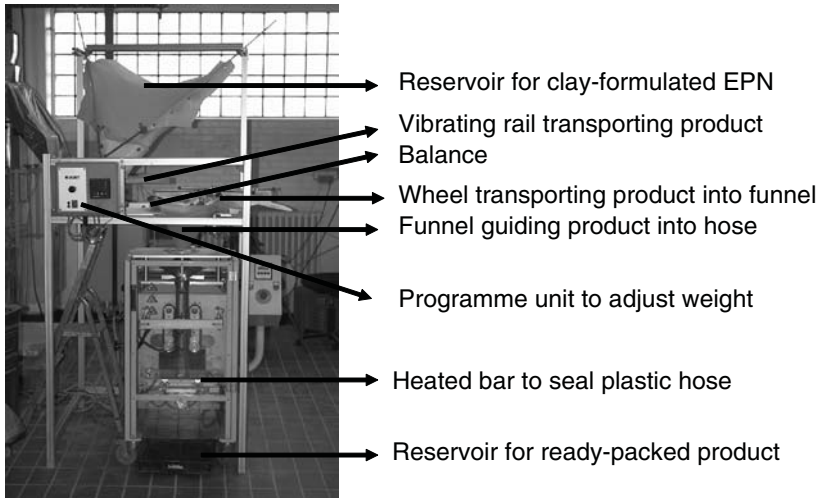


Fig. 15.7. Packing machine used to pack clay-formulated nematodes into plastic bags. Formulated nematodes are dropped on a foil on top of a balance. Once the programmed weight is reached the foil is moved by the wheel into the funnel. The funnel transports the product into the plastic hose. The hose is heat sealed and the bag drops into the reservoir.

Our lower production costs allowed us to offer the nematodes at half the existing market price, but marketing was difficult for us. We were lucky to find a new German distributor, re-natur, who had excellent expertise and experience in the biocontrol market. Offering nematodes at lower costs, they immediately obtained a major market share in the BVW market. Within 5 years, 95% of German tree nurseries had switched to nematodes as the major control of this pest. Another market we developed was the turf market. Results had shown that

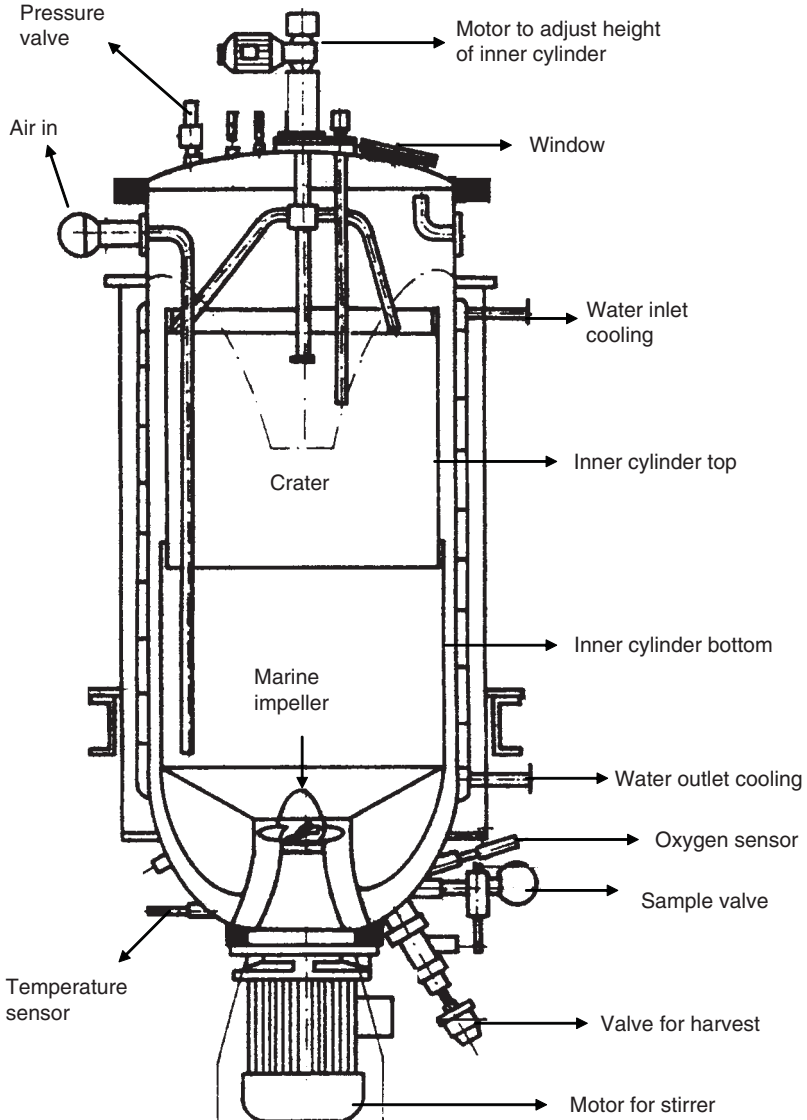


Fig. 15.8. Bioreactor of 3000 litres (Rosenmund, Switzerland). The marine impeller transports the liquid downwards inside the inner cylinder and upwards between the cylinder and the bioreactor wall, forming a crater on top of the liquid phase thus increasing the surface area between gas and liquid.

H. bacteriophora was not able to produce a knock-down effect against turf grass grubs in Germany, but obtained up to 95% control after a period of 8 weeks. It turned out that there was not only a market in professional lawn care and golf but also in the home and gardening market. We produced colourful boxes and flyers (www.e-nema.de), which were meant to attract customers in the non-professional sector.

In the second year, Arne managed to produce *S. feltiae*. This enabled the company to sell products also in the sciarid market. Different to the situation in the UK, where mushroom producers routinely use nematodes to control sciarids, the growers in Germany would not easily accept the product and this has not changed much to date. But in the market for ornamentals, the sales were significant.

The German market is still more than half of the EPN production of the company. Markets in Europe needed exploration. Fortunately, the COST Actions 812, 819 and 850 (www.cost850.ch) had produced a network of scientists in public and private organizations within Europe, and these contacts were valuable for product introduction, primarily in EU member states. Today the company sells products all over Europe and smaller amounts to the USA and Canada.

Nematode Production is a Seasonal Activity

Presently e-nema is the second largest nematode producer after Becker Underwood. Our production capacities were further increased with installation of another internal loop reactor of 6000 litre and one of 10,000 litre equipped with an Intermig impeller, specially designed to produce extreme turbulence to supply high-density bacterial culture with enough oxygen, resulting in a total production capacity of approximately 7×10^{13} DJ per year. However, sales are little more than 5×10^{12} per year. This demonstrates the dilemma of a nematode-producing company: EPNs are a seasonal business and the current production capacity in Europe is much larger than the market potential, resulting in significant undercutting of market prices since 2004. Becker Underwood has double the capacity of e-nema. Besides *S. feltiae*, *Steinernema kraussei* and *H. megidis*, they produce five other nematode species: *Phasmarhabditis hermaphrodita* is produced for control of slugs (see Wilson, Chapter 16 this volume), *S. riobrave* for *Diaprepes abbreviatus*, *S. carpocapsae* for *Hylobius abietis*, *S. glaseri* for grubs, and *Steinernema scapterisci* for mole cricket control. Thus their markets are more diverse and in different climatic zones, whereas e-nema has its focus on the European market. This results in empty bioreactors over the winter season.

Producing More Than Nematodes

In order to optimize productivity of the plant, we planned at the start of the company to produce microorganisms for plant protection either for other companies or to develop our own products. Today e-nema is a major producer of microbial products based on *Pseudomonas fluorescens*, *Pseudomonas chlororaphis*, *Serratia entomophila*, *Serratia plymuthica*, different *B. thuringiensis* and *Bacillus subtilis* strains, *Streptomyces griseoviridis* and *Gliocladium catenulatum*. Contract production is approximately 25% of the total turnover of the company and is likely to further increase should EPN demand not expand.

Biocontrol Production is More Than Just a Monetary Investment

After all the struggles with industry partners, today it is apparent that the start of e-nema was the right way to go. Biotechnology is not comparable to other businesses, where investment is returned within 5–8 years. Particularly in the agricultural biotechnology sector, a return of investment can take 10–15 years. But as e-nema is a family-owned business, the motivation for continuation is not primarily the immediate return of the investment. Fourteen people are employed. Many of us are highly motivated and we want to see our visions put into practice. Venture capital investors would probably have already closed e-nema. Analysing the failure of venture capital enterprises in the biocontrol business, one must conclude that this was not a success. However, most of the family-owned small to medium enterprises like e-nema are still operating. Perhaps venture capitalists need more patience to see these companies succeed. However, patience cannot be expected from them as there are other more lucrative investment opportunities. But the perspectives are good that e-nema will one day pay back the investment as it does not depend on only one product; diversification of contract production into other sectors like aquaculture and cosmetic industries enhances the stability of the company and the probability of success. However, I hope this will be realized before I retire.

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16 A Novel Nematode for Management of Slugs

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Overview: Molluscs are pests for which few biological control agents were available. This is the story of the discovery and successful commercial development of a novel species of nematode with specific activity against slugs.

The Mollusc Problem

Mollusc pests are becoming more of a problem in agriculture and horticulture throughout the world (Barker, 2002) (Fig. 16.1). Terrestrial molluscs are generally referred to as slugs and snails, but there is no scientific definition regarding which is which and animals are called slugs if they have no external shell, or an external shell that is small in comparison to the body. Snails have a large external shell, into which they can usually retract their entire body. Unlike insects, terrestrial molluscs lack a water-retentive cuticle and are thus entirely dependent on availability of water to be active. Thus, in many parts of South-east Asia, aquatic apple snails (*Pomacea* spp.) that have been introduced from Central America are serious pests of rice. Outside this unusual freshwater agricultural habitat, the majority of terrestrial molluscs tend to be most damaging in countries with damp, mild climates. North-west European countries, and particularly the UK and the Netherlands, are 'hotspots' for mollusc pests, but slugs also cause severe crop damage in parts of Australia and New Zealand, Central America and certain parts of the USA and Canada. While slugs have been present in these areas throughout civilization, they have only really achieved the status of major pests in the last 30 years or so. This has been as a result of changes in farming practices, including the banning of burning crop residues in the field, change in crops grown and the move to reduced or no tillage systems for soil conservation. While there are environmental benefits associated with all of these changes, they have encouraged large build-ups of slug populations.

Another reason for the increasing pest status of molluscs is the success of integrated pest management systems to manage many insect pests – a result of the diligence and hard work of the relatively large number of highly trained entomologists. This is very different from the situation for malacologists (people who study molluscs). Very few people are trained in malacology, and those who are



Fig. 16.1. The grey field slug *D. reticulatum* feeding on an oilseed rape seedling.

tend to concentrate on marine molluscs and particularly their taxonomy. Thus, as the pest status of molluscs increased in the UK in the 1970s and 1980s, and farmers started complaining to the government to do something, the government agencies were struck by the fact that there was virtually no knowledge about these animals, their ecology and factors that led them to become pests.

A Slug Research Group is Established

It was against this background that a slug research group was established at the Long Ashton Research Station (now closed), near Bristol in the south-west of England. The group was led by Dr David Glen, an entomologist who had previously worked on control of codling moth in orchards using, amongst other strategies, codling moth granulosis viruses. The group started studying population dynamics, taxonomy and control methods for slugs and soon established a reputation as a centre of excellence in this field. With David Glen's background in microbial control, he soon started looking for funding to investigate the possibility of biological control of slugs, and secured 2 years of funding from the Agricultural Genetics Company (AGC, who later became MicroBio and latterly Becker Underwood) for a speculative search for potential slug control agents. It may seem surprising now that a company would invest a fairly substantial amount of money in a speculative search for slug control agents that so easily could have resulted in nothing. However, this was in the second half of the 1980s, when the biotech boom was well underway, and venture capitalists and many companies were investing heavily in agricultural biotechnology, with what we now know to be unrealistic expectations. As a result of securing AGC funding, I was employed as

a fresh-faced graduate with a Bachelors degree in microbiology. As is usually the case in such degree programmes, the emphasis had been on medical aspects of microbiology and I had no knowledge of invertebrate pathology, other than a vague recollection of *Bacillus thuringiensis* being mentioned in lectures on that genus, in amongst anthrax and food poisoning. To overcome this problem I made a 1-day visit to the Glasshouse Crops Research Institute (also now closed!) in Littlehampton, to talk to members of their thriving invertebrate pathology group. During this visit I spoke to people studying insect viruses, nematodes and fungi, and was fortunate enough to benefit from the wisdom of Denis Burges. Not only did he give me general advice about bacterial pathogens of invertebrates, he also gave me some very useful pointers on how to get started. I returned from the visit feeling somewhat daunted at my lack of knowledge in this field, and rather apprehensive about my chances of success. Certainly there was little information of help in the scientific literature – nearly all references to pathogens of terrestrial molluscs were pretty much anecdotal. The only substantive piece of work was done by the invertebrate pathology stalwart Wayne Brooks during his doctoral studies. This resulted in a very fine piece of work describing the protozoan parasite *Tetrahymena rostrata*, which appeared to offer some promise as a control agent for *Deroceras reticulatum*, the most destructive slug species (Brooks, 1968). This parasite is lethal, can be mass produced and did look promising. However, much later on, when I found this parasite in the UK we determined that, while it was good at killing slugs at Californian temperatures ($> 20^{\circ}\text{C}$), at temperatures typical of UK soils when slugs are a problem ($10\text{--}15^{\circ}\text{C}$) the protozoan had no detectable effect. Apart from Brooks' work, I was starting with a blank canvas. The eventual success of the project was largely a result of being part of a thriving slug research team who were involved in field experiments at many sites throughout the south of England.

The Search for a Slug-specific Pathogen

Slugs are largely subterranean in habit, so in order to study populations, soil samples of known size are taken, returned to the laboratory and slowly flooded over a period of 9 days to force slugs to the surface. Slugs appearing on the soil surface are collected daily. Thus, one of my colleagues would inspect the large number of soil samples every day, and then deliver a similarly large supply of slugs to me, with a mix of species and from a range of sites. The approach I took was simple, just mix the slugs together in boxes under fairly crowded conditions so that any diseases present would spread rapidly through the box – organisms could then be isolated from the cadavers and used to fulfil Koch's postulates. Anybody who has ever tried culturing slugs will realize, however, that keeping slugs together in crowded conditions tends to result in mass slug death irrespective of pathogens and parasites – it appears that slug slime, as it ages, becomes toxic to slugs. This led to much fruitless isolation of bacteria and my development of an agar growth medium based entirely on autoclaved slugs, which created an unforgettable smell.

The Discovery of a Slug-specific Nematode

Approximately 6 months into the project, my luck picked up with the isolation of the nematode we now know to be *Phasmarhabditis hermaphrodita*. Unlike the mysterious and probably non-existent slug pathogenic bacteria I had been attempting to isolate, *P. hermaphrodita* was easy to spot. Slugs infected with this nematode develop a very characteristic swelling in the rear half of their mantle at an early stage of infection (Fig. 16.2), and following death, many large (≈ 3 mm) nematodes can be seen feeding on the cadaver (Fig. 16.3). Once they have devoured what is left of the slug cadaver, the juveniles fail to develop into adults and form non-feeding infective dauer juveniles, much the same as the entomopathogenic nematodes described by Ehlers (see Chapter 15 this volume). The relative ease of culture of *P. hermaphrodita* and the characteristic symptoms caused in slugs made fulfilling Koch's postulates a relatively simple task. While as a microbiologist I was not overly enthralled by the prospect of working with these strange worms, the industrial sponsors were delighted with the discovery.



Fig. 16.2. Healthy (left) and nematode-infected (right) individuals of *D. reticulatum*. This infected slug has an extremely pronounced swelling of the mantle area, where the nematodes initially infect.



Fig. 16.3. *D. reticulatum* that has died following nematode infection, showing nematodes spreading over and feeding on the entire cadaver.

The Nematode Moves to Commercialization

The late 1980s were exciting times for nematode-based biopesticides. AGC were then producing *Steinernema feltiae* in solid *in vitro* culture using Bedding's (1984) pioneering method, and were starting to develop their own methods for liquid cultivation of nematodes, making the prospect of very large-scale *in vitro* culture seem realistic (also see Ehlers, Chapter 15 this volume). In addition, in the UK and much of Europe, nematode parasites have the distinct benefit over all other invertebrate pathogens of being exempt from registration, so commercialization is a more rapid and considerably cheaper process. Following the discovery and preliminary promising results, my contract was extended and I chose to make my studies on *Phasmarhabditis* the basis of a doctoral thesis. As such, I was at least partly involved in all aspects of research and development on this nematode until its eventual release as the product Nemaslug® in 1994 (Fig. 16.4).

The relatively rapid progress to market was helped by the expertise of AGC's nematode production staff and the experiences they had with entomopathogenic nematodes, but this is not to say that the research was without problems. The first of which was identifying the nematode. There were no nematologists employed at LARS, so we sent samples to our sister institute Rothamsted Experimental Station (now Rothamsted Research, a UK agriculture research institute, which is thriving). The key characteristics of this group of bacterial-feeding nematodes are the mouthparts and the male reproductive structures, but as the name implies, the vast majority of *P. hermaphrodita* are self-fertilizing hermaphrodites, with males forming less than 1 in 1500 individuals, and in our strain even fewer. Eventually males were found and the

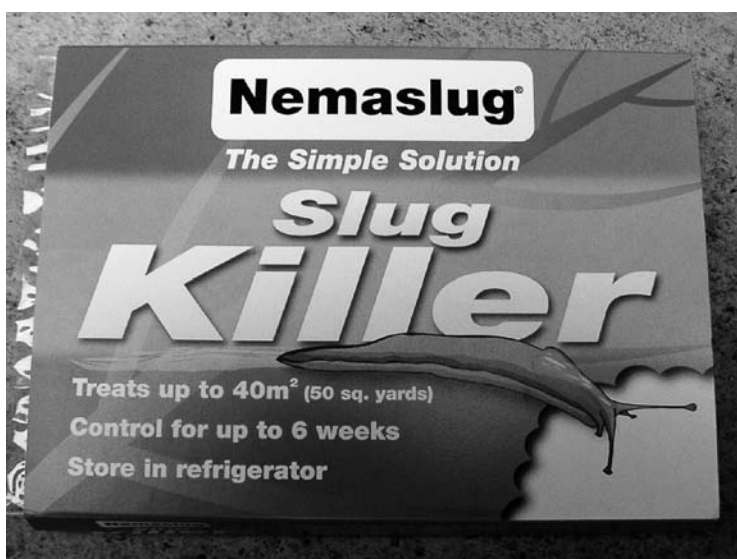


Fig. 16.4. Commercial product Nemaslug® as sold in the UK. This garden-size packet contains 12 million dauer juveniles of *P. hermaphrodita*.

identification made. Once I had demonstrated the feasibility of growing *P. hermaphrodita* in deep liquid culture using a medium based on chopped kidney, all matters of mass production and formulation were left to the staff of AGC. Work at LARS concentrated on investigating dose response relationships, elucidating basic biology of the nematode, investigating host range and demonstrating field efficacy.

As previously mentioned, the slug *D. reticulatum* is by far the most widespread pest slug, and all initial studies were done using this species. However, in most agricultural situations, this species occurs as the dominant pest species but with a sizeable minority of other species, usually from the genus *Arion* – which form an entirely different family of slugs. The host range of *P. hermaphrodita* is fairly broad but much narrower than that of the entomopathogenic nematodes. The nematode kills *Deroceras* spp., small specimens of *Arion* spp. and some larger species from the genus *Milax*. The nematode has the curious ability, particularly in large slugs, to adopt a necromenic life cycle, in which the infective juveniles enter the slug's body cavity and remain dormant there without doing harm until the slug dies, when the juveniles develop and reproduce, feeding on the cadaver. When the nematode enters smaller slugs, it develops directly, causing disease and death of the host. This curiosity resulted in a great triumph for AGC as it allowed them to secure a very tight patent on the use of the nematode as a molluscicide (Wilson *et al.*, 1993). Previous studies on the genus *Phasmarhabditis* done in France in the late 19th century and later in Germany in the 1950s had used the large slug species *Arion ater* and *Limax cinereoniger*. In both these species the nematodes enter the necromenic life cycle, and previous workers had stated categorically that *P. hermaphrodita* did not live parasitically in slugs (Mengert, 1953). Thus, our demonstration that the nematode could parasitize and kill slugs made our discovery sufficiently 'non-obvious' as to allow a patent to be secured. However, while this curiosity afforded patent protection, it is less than ideal because certain larger *Arion* species, particularly *A. lusitanicus*, are serious pests throughout Europe and cannot be controlled with *Nemaslug*.

The Nematode is Capable of Growth on a Wide Range of Bacteria

Much of the early research I did concentrated on investigating the relationship between *P. hermaphrodita* and bacteria. The success of entomopathogenic nematodes both commercially and in nature is attributable to their mutualistic symbioses with Gram-negative entomopathogenic bacteria from the genera *Xenorhabdus* and *Photorhabdus*, and it was assumed the same must be true for *Phasmarhabditis*. This appears not to be the case and no such symbiont has been found. However, the relationships between *P. hermaphrodita* and bacteria make another curious story, which merits a great deal more research. Unlike the entomopathogenic nematodes, which only grow on their own symbionts or closely related congeneric species, *P. hermaphrodita* is capable of growth on a wide range of bacteria, but nematodes grown on different bacteria differ dramatically in virulence. The reasons for this are as yet unknown, but they appear to be unrelated to virulence of the

bacteria alone. A comprehensive review of the biology, production and formulation of *P. hermaphrodita* can be found in Wilson and Grewal, 2005.

Having selected a bacterium (*Moraxella osloensis*) that produced consistently pathogenic nematodes for mass production, we undertook a series of field experiments, ranging from small plots of Chinese cabbage, through lettuce grown in polythene tunnels and on to large field plots of wheat. All early field experiments worked surprisingly well and it appeared that the optimum dose for applying *P. hermaphrodita* was similar to those suggested for entomopathogenic nematodes. Since *in vitro* production yields were similar to entomopathogenic nematodes, this suggested that the economics of selling *P. hermaphrodita* for pest control would be satisfactory in higher value crops. Following publication of the patent in 1992, there was considerable interest in the nematode (all work had remained confidential prior to this) and finally, in 1994, the product was launched into the home garden market, where there was much demand and much scope for selling very expensive nematodes to gardeners keen not to use chemical pesticides.

Shortly after the commercial launch of *P. hermaphrodita*, I left LARS and went to the USA to study entomopathogenic nematodes with Randy Gaulger at Rutgers University. However, since then, *P. hermaphrodita* as a product has gone from strength to strength. The product is now making the transition from being a garden and protected crop treatment to being used in field vegetables, largely as a result of excellent work done by Albert Ester in the Netherlands (Ester and Wilson, 2005), who demonstrated that repeated applications of very low doses give good control, but using one-third of the dose suggested for single applications. Interesting work is also being done in the USA trying to elucidate the interactions between slugs, nematodes and bacteria (Tan and Grewal, 2001), and Becker Underwood are expanding their production facilities and see *P. hermaphrodita* as an integral part of their nematode business.

Many Unknowns Still Await Elucidation

Now I have returned to the UK and am leading my own research group, I have again started working on my favourite nematode, trying to elucidate the many unknowns of its natural history. I firmly believe that we know less about this organism than any other commercially available biocontrol agent. However, in the current funding climate and preference for modern, often technique-driven research, there is little money available for the very basic biological experiments that need to be done, and as long as the product works consistently well, the industry sees no need to fund such work.

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17

A Novel Bacterium for Control of Grass Grub

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Overview: The native New Zealand grass grub *Costelytra zealandica* is one of a few endemic species that have successfully made the switch from a native grassland habitat to the introduced grass and clover pastures. This chapter covers the discovery and commercialization of a microbial biopesticide for this pest based on the bacterium *Serratia entomophila*.

The Search for an Elusive Mortality Factor

My first task in my new job at the Ministry of Agriculture and Fisheries, after filling out the unavoidable forms, was to examine some grass grub larvae. We were looking for an elusive factor X, the cause of grass grub population decline or collapse. The presence of an X factor had been hypothesized by MAF scientist Ray French, who had conducted extensive surveys of this pest. The hypothesis was indirectly strengthened through other researchers who were irritated by grass grub, as after starting long-term population studies they would often find that their research population would disappear. While many factors could be involved in population decline, Ray French was convinced that there would be one king hit to explain the phenomenon.

One of the Worst Scourges of the Pasture Base of the New Zealand Economy

The larvae before me were typical, small melolonthine scarabs. The New Zealand grass grub (*Costelytra zealandica*) is one of over 100 endemic species of Scarabaeidae that radiated in the Gondwanaland remnant isolated from the rest of the world for 60 million years. *C. zealandica* is one of a few endemic species that have successfully made the switch from a native grassland habitat to the introduced grass and clover pastures favoured by farmers since European colonization



Fig. 17.1. Damage caused by the New Zealand grass grub (*Costelytra zealandica*) in New Zealand pastures.

over the last 200 years. The insect certainly has been successful, infesting a million hectares of grassland, where it can reach very high densities (Fig. 17.1). In its original habitat of native tussock and bush, grass grub occurs in small patches and rarely reaches population densities of more than $50/\text{m}^2$. In improved rye grass/clover (*Lolium perenne*/*Trifolium repens*) pastures, pest populations often reach ten times this number and totally devastate the infested grasslands.

Fortunately for pest management, grass grub populations follow a regular cycle in infestation zones where there are no catastrophic climatic effects. Cultivation during preparation of pastures for sowing usually kills most grass grub in the soil. This results in a low initial population in the newly sown pastures. The insect has an annual life cycle, with adults emerging and flying in early summer. In spite of massive flights of beetles, a behavioural quirk assists population prediction. Grass grub females emerge from the soil, release pheromones to attract the flying males, copulate and in an apparent 'home is best' strategy return to the soil in the same location to lay their first batch of eggs. This, coupled with a relatively low fecundity of 10–12 eggs per female, results in predictable population growth over the first 4–5 years in infested pastures (East and Kain, 1982). Following peak populations and damage, the population inevitably declines, and it had always been a point of contention whether decline is due to resource limitation (lack of food for grass grub) or due to French's X factor.

The Discovery of Factor X

The grass grub larvae in front of us were active and appeared normal apart from a clear appearance. We also found that they were not feeding, so our interest was

heightened in these unusual insects. Pathogens were suspected, but cooperation with other scientists gave no indication of the regular culprits (virus, protozoa, bacilli) and for a time rickettsia or some other form of 'difficult' organism was suspected. Frustrated by lack of progress, lab leader Trevor Trought took some larvae to the local vets, who, for lack of other knowledge, cultured microbes from the sick insects and presented us with a Petri plate of bacteria. Smears were added to carrot, fed to grass grub and, Presto!, symptoms were produced, Koch's postulates proven, and amber disease was discovered.

My own background was in economic entomology and establishing damage thresholds and, in these studies, I recall being slightly annoyed by the presence of dead insects, as these would muddy the results. But now, as a new convert, I was enthused by insect pathology. This was helped by Dennis Burges, who convinced me that nothing would beat getting involved in pathology myself, but the initial enthusiasm was assisted by expert advice. Our first simple identifications established the bacteria as non-spore-forming members of the genus *Serratia* (Enterobacteriaceae). Cooperation with Prof. Patrick Grimont, Institute Pasteur, revealed that we had discovered a new species, *Serratia entomophila* (Grimont *et al.*, 1988), which is common in New Zealand soils but has rarely been found outside New Zealand, with the intriguing exception of a few European isolates. Early results indicated that New Zealand pasture soils were populated by two species of *Serratia*, *S. entomophila* and *Serratia proteamaculans*, both of which could occur in pathogenic and non-pathogenic forms, raising questions about the basic genetics of pathogenicity.

Amber Disease Caused by a Novel Bacterium with Unusual Pathology

Grass grub has been an amazing source of pathogens. Through the work of Travis Glare, myself and other colleagues, a list of approximately 30 primary pathogens is known from grass grub (Glare *et al.*, 1993b). Among these amber disease is the most distinct. It is caused by novel bacteria and has an unusual pathology. From our initial experiments, it was evident that disease could be caused by ingestion of pathogenic bacteria, rapidly causing a cessation of feeding and gut clearance, which gave the larvae their amber appearance (Fig. 17.2). A fortunate cooperation agreement between New Zealand and Germany gave me the opportunity to work at the Institut für Biologische Schadlingsbekämpfung (Institute for Biological Control) of Darmstadt Germany, the site of much pioneering research in insect pathology. Alois Huger recognized the unusual characteristics of amber disease, and together we initiated a histopathological study. Surprisingly, there was no obvious damage to the epithelial cells, but gut colonization was marked by bacterial adhesion to the cuticular membranes of the foregut and crop (Jackson *et al.*, 1993). Once feeding ceases, the gut becomes a vesicle for bacterial growth while the insects remain in the chronic non-feeding phase of disease. The infected insects remained in a non-feeding state for weeks to months until weakened gut tissues ruptured, leading to bacterial invasion and



Fig. 17.2. Healthy grass grub larva (left) and amber-diseased larva (right) with remnants of carrot food supply provided for the previous 24 h, illustrating that diseased larvae cease feeding.

septicaemia (Jackson *et al.*, 2001). The exact cause of pathogenicity remains elusive but may be uncovered by molecular studies on both bacterial and insect gene expression being currently carried out.

Bacterial septicaemia is accompanied by a rapid breakdown of the cadaver and release of bacteria back into the soil. Study of individual bacteria within the soil matrix is extremely difficult for most species but is made possible through one quirk in the biology of the genus *Serratia*. Unlike most soil bacteria, *Serratia* spp. can grow on media rich in thalium salts, and caprylate thalious agar provides an excellent selective medium for isolation of *Serratia* spp. from soil and is key to understanding the ecology of applied species. In research led by Maureen O'Callaghan, we have used a combination of selective media, biochemical tests, phage typing and molecular typing systems (O'Callaghan and Jackson, 1993; Claus *et al.*, 1995; O'Callaghan *et al.*, 1997) to track individual strains of bacteria in the soil and elucidate the post-release ecology of the bacterium in a detailed manner. Application of the bacteria to the soil surface leads to rapid death of the bacteria, but if drilled into the soil there is little loss of viability during application. Once in the soil, bacterial numbers, in the absence of larvae, decline by one log about every 6 weeks, depending on soil conditions. In the presence of grass grub larvae, pathogenic strains are maintained by bacterial production through the infected insects. In general, a pasture at the height of an epizootic of amber disease will have a mean population of 10^4 – 10^5 pathogenic bacteria/g soil. Once the grass grub population has declined to low levels it appears that pathogenic strains are unable to compete with non-pathogenic, and the population balance will shift back to the non-pathogenic bacterial strains, which are a normal component of the soil microflora (O'Callaghan *et al.*, 1999).

New Zealand pastures have been an excellent laboratory for study of the insect/disease interaction. As these occur in the soil, sampling is a laborious job, where Richard Townsend has led the team to determine population fluctuations

and impact of disease application. Bacteria are applied at the rate of 4×10^{13} cells/ha, which is sufficient to establish bacteria in the soil and initiate the first cycle of disease (Fig. 17.3). Key to the success of *Serratia* pathogens is bacterial recycling. Once established, pathogenic bacteria will persist until the grass grub population declines to low levels or is killed by adverse soil conditions. Modelling by Nigel Barlow suggested that grass grub are held at 50% of their potential density by natural epizootics and indicated ways of optimizing bacterial use (Barlow 1999). The soil ecology of grass grub pathogenic *Serratia* was further modelled by Godfray *et al.* (1999), who highlighted the importance of saprophytic growth of bacteria in the soil.

The quest to understand the basis of pathogenicity led to a long and useful cooperation with genetics professor Kris Mahanty at the University of Canterbury. Kris and his students isolated phages from *S. entomophila*, which, while originally found in failed fermentations, have proven invaluable for strain typing in ecological and commercial evaluation studies. The team also initiated studies on the genetics of the disease. A major breakthrough came with the discovery that pathogenicity genes were plasmid borne (Glare *et al.*, 1993a; Grkovic *et al.*, 1995; Hurst *et al.*, 2000). Subsequent studies led by Mark Hurst have shown that pathogenicity is encoded by distinct amber-causing and anti-feeding genes (Hurst *et al.*, 2000, 2004). Interestingly the *Serratia* pathogenicity genes show a strong homology to those found in nematode-associated *Photorhabdus luminescens*, suggesting a new family of insecticidal toxins associated with these bacteria (Hurst *et al.*, 2000; Waterfield *et al.*, 2001). The mode of action of the bacterium also encompasses some unusual properties, with early infection inducing a shut down of digestive enzyme synthesis (Jackson, 1995; Jackson *et al.*, 2004).



Fig. 17.3. Early test applications of *Serratia entomophila* for grass grub control by the microbial control team of AgResearch in Canterbury, New Zealand.

The Development of a Biopesticide

The isolated bacteria could be cultured through *in vitro* fermentation, and bacteria produced in this manner could be applied back to the soil causing disease (Jackson *et al.*, 1986), raising the potential for use as a biopesticide. Enthusiasm certainly outweighed knowledge in the early stages of development. Field tests indicated the potential of the bacterium but, as the organism was novel, safety testing was a high priority. *S. entomophila* passed Tier I tests and became the first indigenous microorganism registered for insect control in New Zealand (Jackson *et al.*, 1992). However, high bulk and short shelf-life of the original prototype product posed challenges for commercial exploitation.

Finding an appropriate commercial partner for biopesticide development can be as much of a challenge as technical development. In the 1980s, Monsanto initiated a programme for biopesticide development and with the local representatives, led by Murray Willocks, *S. entomophila*, as the product Invade™, became Monsanto's first and probably only biopesticide launched on to the market (Jackson *et al.*, 1992). While efficacy was high, needing only 1 litre of fermenter product to treat a hectare of pasture, there were some difficulties. Unlike conventional chemicals, Invade™ had a limited shelf-life and, for successful results, had to be placed in the soil in a large volume of water using modified seed drills. Monsanto addressed this problem by establishing distribution points and registered contractors. But potential market penetration was limited by restricted outlets, and changes in emphasis led to Monsanto withdrawing from the product.

Invade™ continued to be produced and marketed by a range of companies in subsequent years, treating several thousand hectares annually, but a new approach was needed to gain greater market acceptance. Formulation appeared to be a key, and research led by Von Johnson resulted in a new flowable granular product that could be stored for considerable periods in ambient conditions without deterioration (Johnson *et al.*, 2001). The new formulation has been developed as part of a joint venture (EnCoate Ltd) between our research institute, AgResearch, and one of New Zealand's largest fertilizer companies, Ballance Agri-nutrients Ltd (Fig. 17.4). The granular formulation, Bioshield™, has now been marketed for 2 years and is making good progress in the pastoral sector. The stable formulation can be sold directly to individual users, which provides greater diversity in application and allows farmer innovation to come into play. It also needs greater farmer education in how to use the product, but like its predecessor Invade™ the bacteria can be used to good effect and forms the basis of grass grub management in some grass grub prone regions.

Lessons Learned

The *Serratia* experience has been a programme that we stepped into naively but fortunately have managed to steer to a successful conclusion. Protection of young pasture from attack by grass grub proves the point that bacterial efficacy rather than resource limitation drives the population dynamics of the insect. Through a



Fig. 17.4. Large-scale production of Bioshield™ granules (10 tonnes/h) by Ballance Agri-nutrients in New Zealand.

mixture of basic science, ecology and product-development funding we have managed to maintain a continuous research programme, even though at times a little thin, on *Serratia* pathogens for the past 25 years. I have discovered a lot about the bacterium, the grass grub and myself! *Serratia* spp. are capable of holding a range of insect toxins, which may provide important opportunities for the control of other pests. More research is needed in this area. We have shown that bacteria can be bulk produced and applied to establish and thrive in the presence of their host. The grass grub remains a pest in New Zealand, but for farmers Bioshield™ provides an effective, safe, 'green' management option, supporting production of chemical-pesticide-free agricultural produce from New Zealand. And for myself – I have realized that a simple observation of an unusual phenomenon can lead to a stimulating career in insect pathology. As the *Serratia* pathogenicity genes join the wider world of the *Photorhabdus* TC complex, the research goes on.

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18

How Early Discoveries about *Bacillus thuringiensis* Prejudiced Subsequent Research and Use

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Overview: *Bacillus thuringiensis* is the most successful and widely used microbial biological insecticide to date. Research has focused on the insecticidal properties of this bacterial group. As a result, its other potential roles may have been overlooked. This chapter examines what other ecological roles or benefits these organisms may have or provide.

***Bacillus thuringiensis*: an Insecticidal Bacterium . . . or is It?**

B. thuringiensis is a gram-positive, rod-shaped, spore-forming bacterium, characterized at the species level by the production upon sporulation of a parasporal inclusion body, the crystal. This crystal is made of proteins, the Cry and Cyt proteins, some of which exhibit specific insecticidal activities on larvae of economically important pests. The mode of action of the Cry protein has been deciphered to some extent. The crystal is ingested by susceptible insect larvae and dissolves in the alkaline midgut. Protoxins, inactive Cry proteins, are released and activated by specific proteases. The activated toxins bind to specific receptors on the cell midgut and undergo conformational changes to allow insertion in the membrane, where they form ion channels, leading to osmotic cell lysis and ultimately larval death (for reviews on the *B. thuringiensis* mode of action, see Rajamohan *et al.*, 1998; Schnepf *et al.*, 1998; de Maag, 2003). As a follow-up to their insecticidal activities, *B. thuringiensis* strains have been developed commercially and formulated as insecticides for the control of lepidopteran, dipteran and coleopteran pests. *B. thuringiensis* is today by far the most successful commercial biopesticide.

History of *B. thuringiensis*

A century ago, Ishiwata was investigating the cause of a disease, a severe flacherie, that was killing silkworms (*Bombyx mori*) in rearing facilities in Japan,

first at the Tokyo Sericultural Institute and then at the Kyoto Sericultural Institute. He isolated a bacterium, a 'bacillus', and proved it to be the causal agent of the disease. He named the disease 'sotto-byo' – sudden collapse disease – and the causal agent 'sotto-byo-kin'. A decade later, Berliner, while investigating the cause of diseased granary populations of Mediterranean flour moth (*Anagasta kuehniella*) larvae, isolated a similar bacterium. Because flours in which the insect larvae were found were from the German province of Thuringia, he named the bacterium *Bacillus thuringiensis*, using the formal Latin binomial scientific nomenclature (for a review on the history of *B. thuringiensis*, see Beegle and Yamamoto, 1992).

The bacterium would be re-isolated again from the same diseased insect species by Mattes in 1927. Based mostly on this latter isolate, the next few years would see the first attempts at using *B. thuringiensis* as a control agent against an economically important insect pest, the European corn borer, *Ostrinia nubilalis*, in parts of Europe. Sporeine, the first *B. thuringiensis*-based commercial formulation was developed and produced in France as early as 1938. Several more *B. thuringiensis* formulations were subsequently developed in Eastern Europe in the 1950s. Thuricide was developed in the USA in the mid-1950s. By the late 1960s, about 150 *B. thuringiensis* strains had been isolated, the vast majority from diseased lepidopterans or from materials associated with them. A paradigm was being established: *B. thuringiensis* was a bacterial species with insecticidal activity against lepidopterans. Several methods were proposed for the classification of the increasing diversity of the *B. thuringiensis* strains. H-serotyping, the serological reaction to the *B. thuringiensis* flagellar antigens, was established as the method of choice. In the late 1960s, a total of nine serotypes was known.

Dulmage and Beegle were the first to establish an extensive screening programme for novel *B. thuringiensis* strains. In 1970, Dulmage isolated a novel *B. thuringiensis* strain from a mass-reared colony of the pink bollworm, *Pectinophora gossypiella*, which he named HD-1. Assays showed that this isolate was much more potent against economically important lepidopteran pests than all other *B. thuringiensis* strains used in commercial products. Dipel, a *B. thuringiensis* HD-1 based formulation, was born soon after. The success of the HD-1 isolate, a serovar *kurstaki* strain, prompted the targeted screening of available *B. thuringiensis* isolates on a variety of insect pests, in the hope of identifying yet more potent strains specific for a given target. Of course, these targets were all lepidopterans. In the mid-1970s, however, a screening programme for mosquito pathogens was established in Israel. This led to the isolation of a novel strain of *B. thuringiensis* from a dense population of *Culex pipiens* in a small pond. It would soon be classified as a new serovar, *israelensis*. The *B. thuringiensis*/anti-lepidopteran paradigm was revisited to include dipterans. In the early 1980s a novel strain of *B. thuringiensis* was isolated from a dead pupa of the yellow mealworm, *Tenebrio molitor*, a coleopteran, and proved to have anti-coleopteran activity. This was a serovar *morrisoni* strain, unique in its anti-coleopteran activity and referred to as pathovar *tenebrionis*. The paradigm was expanded to include other insect orders: *B. thuringiensis* was an insecticidal bacterial species.

These discoveries contributed to the establishment of several more extensive screening programmes for novel *B. thuringiensis* strains expressing novel insecticidal, pesticidal and biological activities. It is estimated that by the late

1990s, more than 60,000 *B. thuringiensis* strains, grouped into more than 82 serovars, 69 serotypes and 13 sub-antigenic groups, were kept in various collections worldwide. Activities against insects in the Hymenoptera, Homoptera, Hemiptera and Mallophaga orders were claimed in various patents, but have yet to be fully described in the scientific literature. Outside of the insect world, however, activities against Nematoda and Protozoa were reported in the scientific literature.

***B. thuringiensis*-based Commercialized Formulations**

To be used as a microbial pest control agent, *B. thuringiensis* needs first to be produced by fermentation (for reviews on the production of *B. thuringiensis*-based commercial products, see Burges, 1998; Couch, 2000; Glare and O'Callaghan, 2000). It multiplies as vegetative cells and enters sporulation, during which parasporal crystals are synthesized. The spores and crystals are released following cell lysis. This lysed culture contains the active ingredient and could theoretically be used directly for the control of insect pests. Results, however, would be inconsistent and not optimized. Several additional steps are required to transform *B. thuringiensis* into a commercial formulation. The biomass is harvested and may or may not be dried. This is the technical-grade unformulated powder or paste, depending whether it has been dried or not, that will be used as the active ingredient in *B. thuringiensis*-based formulated products.

The formulation aims at improving the shelf-life of the product, making it easier to handle and apply, while enhancing its deposition and efficacy, and increasing its persistence. Formulated products may contain microbial growth inhibitors, spreader-stickers, surfactants, UV protectants, etc. Products can be formulated as dried wettable powders, water-dispersible granules, dusts, granules, briquettes, pellets, etc., or as liquid aqueous or emulsifiable oils. The range of formulations available is a reflection of both the needs and the range of habitats of the target pests. Formulations with high concentrations of active ingredients were developed for forest pests, where aerial spraying to cover large areas is done by experts and the cost for aerial spraying is an incentive in reducing the volume sprayed. Conversely, in agriculture, formulations designed for long-term storage and ease of use are usually diluted in large volumes at the site of application by local producers and are compatible with local spraying equipment. Specific formulations were designed for the control of mosquito larvae, which feed in water.

The efficacy of *B. thuringiensis*-based formulations suffers from limitations. Their insecticidal activity decreases rapidly following spraying owing to UV crystal degradation, rainfall, etc. In agriculture, most formulations lose 50% of their activity within 24 h following spraying. Consequently, several applications must be done to achieve adequate pest control and, owing to its short half-life, timing of application becomes critical. The encapsulation technology, a process whereby a chemical pesticide is coated with synthetic polymers, has been used initially to protect chemical pesticides from degradation in the environment or, alternatively, to provide controlled release of the active ingredient. Similar technologies

were developed for *B. thuringiensis*. *B. thuringiensis* was encapsulated with clay, UV-absorbing compounds, chemical polymer matrices and in various starches and biopolymer matrices. These starches and biopolymer matrices act as stickers to provide somehow improved adhesiveness to the plant leaves. Presumably, by entrapping the active ingredient, they also retain the insecticidal activity through better protection against desiccation, sunlight, heat and the damaging effects of UV light. Using a different technology, slow-release formulations were developed for use in water for the control of mosquitoes. Certainly, engineering *B. thuringiensis* crystal genes in plants is another approach, which has been favoured by several companies and organizations for improving the delivery, efficacy and field life of *B. thuringiensis*. These, however, are not covered in this chapter.

A Decade of *B. thuringiensis* Research at St-Jean-sur-Richelieu

I first started working on *B. thuringiensis* in the early 1990s. As a newcomer to this field, my early line of research was established in full accordance with the paradigm. It appeared to me that at least two factors were limiting the use of *B. thuringiensis*: (i) lack of strains active against economically important pests beyond the sempiternal lepidopterans; and (ii) formulations with limited efficacy.

My first task was to establish a survey of the distribution of *B. thuringiensis* in Quebec soils. I received more than 300 soil samples from all over the province. Analyses of these soils revealed that most contained *B. thuringiensis*. I also called upon colleagues within Agriculture and Agri-Food Canada – the Canadian Department of Agriculture – to send me invertebrates, any invertebrates – insects, spider mites and other arthropods, nematodes, molluscs, etc. – that had died of unknown causes that they had encountered, whether in rearing facilities or not. I was to screen them for *B. thuringiensis* in the hope of isolating novel strains that might express novel insecticidal or pesticidal activities. Soon our collection of *B. thuringiensis* contained about 250 isolates, many of them of the *kurstaki* variety. Clearly, the diversity was limited but some strains would turn out to be quite interesting as experiments would show.

Next, we set to conduct a series of bioassays on insects and other invertebrates for which no active *B. thuringiensis* strains had been reported. We purposefully chose to ignore lepidopterans, dipterans and coleopterans. Some of our strains were isolated from the tarnished plant bug, *Lygus hesperus* (Hemipteran), from the grey slug, *Deroceras reticulatum* (Mollusca: Gastropoda), or from the spider mite, *Tetranychus urticae* (Arachnida). All three are clearly economically important pests. A colony of each was established. In addition, we also established a colony of the free-living nematode, *Caenorhabditis elegans*. Two colonies of the beneficial insects, the honeybee, *Apis mellifera* (Hymenoptera), and ladybird beetle, *Harmonia axyridis* (Coleoptera), were established for the assay of both the spectrum of action and the effects on beneficial insects. Finally, a colony of the house fly, *Musca domestica* (Diptera),

was established to serve as a target for the assay for the presence of another *B. thuringiensis* toxin, the β -exotoxin.

Novel Products and Uses Arise

Bioassays conducted with *B. thuringiensis* strains isolated from dead *D. reticulatum* showed some of them to be lethal. Yet, they showed no activity against other tested targets. A collaboration was established with a small Canadian firm, AEF Global, in an attempt to develop the strain into a viable commercial product. After several years of research, an experimental product has now entered the registration pipeline in Canada.

Bioassays were conducted with *B. thuringiensis* strains isolated from dead *L. hesperus*. None of these turned out to be lethal. Additional bioassays conducted with *B. thuringiensis* obtained from two public collections, the 'Laboratoire des bactéries entomopathogènes' at 'Institut Pasteur' in Paris, France, and the *Bacillus* Genetic Stock Center at Ohio State University, Columbus, Ohio, led to the identification of strains active against *L. hesperus* (Wellman-Desbiens and Côté, 2004) or *C. elegans* (Bélair and Côté, 2004).

Bioassays conducted with *B. thuringiensis* strains isolated from dead *T. urticae* showed no acaricidal activity. Yet, preliminary analysis of one of these bacterial strains revealed that it was unusual enough to warrant further investigations: the spore and crystal were tightly coupled to each other, even in fully lysed cultures; the crystal was spherical and made of a single protein population of 83 kDa in size. The gene encoding this protein would turn out to be homologous to the so-called 'Cry31Aa1 parasporin', with specific cytotoxic activity against some human cancer cells. Our protein is now called Cry31Aa2. Assays on mammalian cells have since confirmed the specific cytotoxic activity on some human cancer cells. A patent application was filed (Côté *et al.*, 2005) and further characterization of the strain is in progress (Jung *et al.*, in press).

In addition to the identification of novel targets, we also worked on the development of *B. thuringiensis*-based formulations. Some stakeholders expressed an interest in starting a small company, AEF Global, that could develop and produce *B. thuringiensis* formulations and I became involved in the process. The first step was to develop a fermentation medium that would be economically viable while providing excellent fermentation yields. The second step was to develop fermentation products. This fruitful collaboration led to the development and registration of seven products in the last few years: Bioprotec™ technical, a *B. thuringiensis* var. *kurstaki*-based technical powder that would serve as the active ingredient in subsequent formulations; Bioprotec™, an aqueous formulation developed for use against lepidopteran pests in forestry; Bioprotec™ CAF, an aqueous formulation for use against lepidopteran pests in agriculture; Bioprotec™ HP; a high-potency aqueous formulation; Bioprotec™ ECO an aqueous formulation for domestic use; Bioprotec™ XHP, an extra-high-potency aqueous formulation; and Bioprotec™ 3P, a dustless dry flowable granule with increased persistence under UV light and rain, potency and protection for use against lepidopteran pests in agriculture.

Reflections on Earlier Discoveries in *B. thuringiensis* Research

It is interesting to reflect on the history of *B. thuringiensis* as presented very briefly above, to realize not only how some discoveries have driven subsequent research, notably mine, but also how some conclusions drawn from early discoveries may have been stretched. Certainly, the earlier isolated *B. thuringiensis* strains mentioned above were insecticidal, at least for the insects assayed. Likewise, later directed screening for *B. thuringiensis* strains from diseased insects, and materials associated with them, was likely to yield insecticidal strains. The picture became more complex when screening programmes were expanded to include sources other than diseased insects. *B. thuringiensis* strains were isolated from a variety of different environments: certainly insectaries and insect-associated material – stored grain and stored products – but also from soil samples worldwide including, perhaps surprisingly, Antarctica, cultivated and uncultivated lands, lands associated with insects, lands not associated with insects, forests, the phylloplane and other plant material, sludge of waste-water treatment plants, animal faeces, etc. Far from being restricted to insects and insect-related material, *B. thuringiensis* was ubiquitous (Martin and Travers, 1989). In addition, as the number of strains and diversity of serovars grew, it appeared that the majority of *B. thuringiensis* strains did not exhibit insecticidal activities, at least on the insects against which they were assayed.

Is *B. thuringiensis* Really an Insecticidal Species?

The findings outlined above open the door to several questions:

- Does *B. thuringiensis* really qualify as an insecticidal species, with the emphasis here being on the word ‘species’?
- What is the normal habitat of *B. thuringiensis*?
- What is the role of the crystal?

One would assume that to qualify as insecticidal, a bacterial species must have a large number of strains with known insecticidal activity. To test whether a bacterial species is indeed insecticidal, one would start by assaying a large number of bacterial strains on a large variety of insects. If most, if not all, strains show insecticidal activity, the species could be deemed insecticidal. As described above, the *B. thuringiensis* story has followed a different approach in presenting it as an insecticidal species. Directed screening for pathogens from diseased or dead insects that led to the recovery of *B. thuringiensis* yielded insecticidal strains. The question, however, is not whether *B. thuringiensis* strains are insecticidal but whether the species is? Given the large number of non-insecticidal strains, *B. thuringiensis* hardly qualifies, at the species level, as being insecticidal. *Bacillus anthracis* qualifies, at the species level, as the anthrax-causing agent because most *B. anthracis* strains cause anthrax. Similar reasoning does not hold true for the *B. thuringiensis* species. This is much more than simply a matter of semantics,

since, as we have seen, qualifying *B. thuringiensis* as an insecticidal species has had tremendous implications in driving the research associated with it: screening programmes for *B. thuringiensis* strains from different sources, assays on insects, development of insecticidal formulations, study of the mode of action on susceptible insects, etc.

Whereas the paradigm has been highly successful in generating the vast diversity of *B. thuringiensis* strains, serovars, crystal proteins and insecticidal and pesticidal activities known today, in driving the development of *B. thuringiensis*-based commercial insecticides, in leading to the deciphering of the mode of action in insects, etc., it may have been counterproductive in limiting the amount of research and the innovation in the non-insecticidal or non-pesticidal areas. Whereas several laboratories and companies have established screening programmes for novel *B. thuringiensis* strains, very much in line with previous research and with the insecticidal paradigm, there is only one group worldwide that has assayed an extensive number of strains on human cancer cells in the hope of developing anti-cancer therapeutics (Mizuki *et al.*, 2000).

The actual role of *B. thuringiensis* in the environment is still open to debate (for reviews on *B. thuringiensis* ecology, see Meadows, 1993; Glare and O'Callaghan, 2000). In accordance with the paradigm, it has been proposed that *B. thuringiensis* might be a member of the phylloplane microflora and might protect the plant against insect pests. It has also been proposed that *B. thuringiensis* is a normal soil bacterium and lives in soil-dwelling insects and nematodes. Yet, in the environment, *B. thuringiensis*-caused epizootics in insects are rare. Epizootics have been recorded mostly in rearing facilities, with some exceptions. In addition, *B. thuringiensis* recycles poorly in insect populations in the environment. Spraying of a *B. thuringiensis*-based commercial formulation will be effective at controlling the target pest if done correctly, but re-infection is not expected and repeated applications in the same or consecutive years are usually required for control of insect pests. In addition, *B. thuringiensis* is often found in the environment in the absence of insects. Is there room for *B. thuringiensis* and the crystal outside the insecticidal paradigm? Hasn't the time come for a reappraisal of the '*B. thuringiensis*/insecticidal paradigm at all cost'?

Although *B. thuringiensis* is ubiquitous in the environment, as shown above, whether it is a normal soil or phylloplane bacterium should also be questioned. The ubiquity of *B. thuringiensis* is likely to be a testimony to the resilience and survival of its spore rather than an indication of the normal habitat of the vegetative cell. Indeed, many screening programmes include a heat-shock to positively select sporulating microorganisms, including *B. thuringiensis* spores. The fact that spores can be ubiquitous is not as surprising in itself as it might have looked, given the very nature of the spore, and it is certainly not an indication of the normal habitat of the *B. thuringiensis* vegetative cells. Interestingly, monitoring of *B. thuringiensis*' fate following spraying programmes indicates that the number of spores rapidly decreases during the first few weeks and then remains stable for months. Equally interesting, the addition of *B. thuringiensis* vegetative cells to untreated soil shows that not only do they fail to establish or multiply but they disappear within a few days. Is *B. thuringiensis* truly a normal soil bacterium?

Perhaps the story becomes even more complex when *B. thuringiensis* is regarded as a member of the *Bacillus cereus sensu lato* group. This group also includes *B. anthracis*, *Bacillus weihenstephanensis* and *Bacillus mycoides*. For the purpose of discussion, it is interesting to point out that *B. cereus*, *B. thuringiensis* and *B. anthracis* are nearly indistinguishable at the genotype level (Helgason *et al.*, 2000). Indeed, several studies have indicated that all three species should be grouped in a single species, *B. cereus*. The presence of the parasporal crystal is the most distinctive criterion for the discrimination of *B. thuringiensis* from *B. cereus*. Capability to cause anthrax is the most distinctive criterion for the discrimination of *B. anthracis* from *B. cereus*. The crystal proteins are plasmid-encoded. Likewise, the anthrax toxin and capsule genes are plasmid-encoded. Plasmid curing of *B. thuringiensis* or *B. anthracis* would render them indistinguishable from *B. cereus*. This opens the door to yet more questions: are some *B. cereus* strains found in the environment former *B. thuringiensis* that might have lost their plasmids? The question remains wide open as to the actual natural habitat of the *B. thuringiensis* vegetative cells, with the emphasis here being on 'vegetative cells'.

The actual role of the crystal is still open to debate. The crystal accounts for up to 35% of total proteins in a sporulating *B. thuringiensis* cell. It seems unlikely that the process by which *B. thuringiensis* diverts so much energy and nutrients to the production of this crystal would have been retained had it not conferred some selective advantage. Within the paradigm framework, *B. thuringiensis* is an insecticidal bacteria and the crystal confers an advantage to the bacteria by allowing it to multiply in a susceptible insect. Yet, as mentioned above, some crystal proteins are specifically cytotoxic to some human cancer cells. If the paradigm is in need of reappraisal, as hopefully has been shown here, then the crystal might have some biological role(s) to play other than, or in some cases in addition to, killing insects. From a bacterial evolutionary perspective, it has been proposed that the crystal might act as a reservoir of nutrients to favour germination of the spore (Martin, 1994). It is a refreshing hypothesis worthy of further investigation.

Current *B. thuringiensis* Research at St-Jean-sur-Richelieu

Our work on *B. thuringiensis* has now moved away from the screening of strains expressing insecticidal or pesticidal activities and the development of formulations. We are now pursuing the characterization of Cry31Aa2, which expresses cytotoxic activity against specific human cancer cells. As a complement to H-serotyping, we are now adapting or developing typing methods for the classification of the wide diversity of the *B. thuringiensis* strains: ribotyping (Joung and Côté, 2002), heterogeneity of the 16S-23S Internally Transcribed Spacer (Xu and Côté, 2003), relationships between serotyping and nucleotide sequences of the flagellin genes (Xu, and Côté, 2006), etc. We are also interested in the definition of 'species' for *B. thuringiensis*. The formation of a crystal is the single most significant distinguishing taxonomic feature of the species *thuringiensis*. Yet, based on ribotyping, some *B. thuringiensis* serovars appear phylogenetically distant from the bulk of the *B. thuringiensis* serovars (Joung and Côté, 2002). This raises yet another question: are all *B. thuringiensis*, *B. thuringiensis*? We

have also initiated some work on whether the crystal can be a source of nutrients for the germination of *B. thuringiensis* spores. Somehow, my decade-old applied-type of research, novel strains, novel formulations, has redirected itself into a more fundamental type as questions emerged. Perhaps, after some answers are found to some of the above questions, my research will again redirect itself and hopefully transform some of the newer findings into applied products.

Conclusion

The fact that *B. thuringiensis* has long been, and still is, presented as an insecticidal species is much more a reflection of the way concepts in science, paradigms, may sometimes be established, based, in my mind, on a few incidental observations rather than by thorough scientific investigations. Yet, based on a small number of strains, *B. thuringiensis* has become the most successful commercial biopesticide today. However successful, research on *B. thuringiensis* should not limit itself to the insecticidal paradigm. Available or novel *B. thuringiensis* strains may very well carry yet to be revealed novel insecticidal, pesticidal and biological activities. Eventually, perhaps, *B. thuringiensis* will be regarded, from a biotechnology standpoint, not as an insecticidal species but rather as a pool, as a source of a wide variety of toxins potentially expressing cytotoxic activities on an equally wide variety of target cells. In the meantime, from a bacteriology standpoint, progress should have been made regarding the normal habitat of *B. thuringiensis* and the true role of its crystal.

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19

Development of Resistance to the Biopesticide *Bacillus thuringiensis kurstaki*

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Overview: Continued use of the microbial insecticide, *Bacillus thuringiensis kurstaki* (*Bt*), ultimately depends on our ability to prevent resistance from developing in pest populations. Reports of *Bt* resistance in field pest populations have been infrequent and largely restricted to *Plutella xylostella*. In contrast, populations of *Trichoplusia ni* present in commercial vegetable greenhouses have been shown to repeatedly develop *Bt* resistance. This finding has provided a unique opportunity to study the evolution of *Bt* resistance, and illustrates how a pest's environment can amplify resistance evolution.

Introduction

The microbial insecticide *Bacillus thuringiensis kurstaki* (*Bt*) has become the mainstay of non-chemical control of lepidopteran pests (see Côté, Chapter 18 this volume). However, the continued use of *Bt* depends on preventing the evolution of resistance in target pest populations (Ferré and van Rie, 2002). Insect pests have continually evaded chemical insecticides through the development of resistance. Resistance problems often occur within a few years following an introduction of an insecticide. With respect to *Bt*, laboratory experiments involving over 16 pest species have demonstrated that resistance to *Bt* can be selected for (Tabashnik, 1994). Nevertheless, despite over 30 years of use, only one pest species, the diamondback moth (*Plutella xylostella*), has been reported to have developed significant resistance to *Bt* outside the laboratory (Ferré and van Rie, 2002). Hence, it has been commonly assumed that resistance will rarely evolve in insect populations in response to applications of microbial controls. This case study, however, highlights the second occurrence of *Bt* resistance in an agricultural situation – the resistance of cabbage loopers, *Trichoplusia ni*, in vegetable greenhouses in British Columbia, Canada – and discusses how greenhouse environments can contribute to resistance evolution.

Management of Cabbage Looper Population in Greenhouses

Commercial greenhouse vegetable growers in British Columbia (BC), Canada, rely heavily on *Bt* for the control of cabbage loopers. *T. ni* is a significant pest in a wide variety of crops in the Fraser Valley region of BC and is currently the only lepidopteran pest of economic concern in commercial vegetable greenhouses in BC. Other biological control agents, such as the parasitoid wasp *Trichogramma brassica*, and the hemipteran predator *Podisus maculiventris*, have been used for *T. ni* control, with variable results. The technical expertise required to successfully utilize these biocontrol agents, high costs and, in some cases, inconsistent production of biocontrols by suppliers have been limiting factors in the success of these two biological control agents. Growers prefer the low expenses and rapid results associated with applying a microbial insecticide and have, therefore, often used *Bt* almost exclusively for *T. ni* control in greenhouses.

For greenhouse growers, the major threat to successful cabbage looper management was the potential for *Bt* resistance to evolve. In 1999/2000, growers began to report poor control of cabbage loopers following *Bt* applications. In response to inadequate *Bt* efficacy, growers increased the frequency and rate of *Bt* applications. These measures eventually caused growers to pursue emergency applications for registration of chemical control products. At the time, the reason for failing *Bt* efficacy was uncertain, and speculations arose about the quality of *Bt* products. However, reports of poor effectiveness of an insecticidal spray are often the first signs of resistance development in a pest population (ffrench-Constant and Roush, 1990).

On the Road to Discovery of Resistance

In 2000, a 3-year survey was initiated to determine if genetic resistance to *Bt* was present in *T. ni* populations. Populations of *T. ni* infesting commercial vegetable greenhouses and local broccoli fields were sampled for resistance and were compared to a susceptible laboratory population. Several greenhouse populations were surveyed multiple times within a growing season to monitor the rate at which resistance developed within 1 year in response to grower *Bt* sprays. The survey demonstrated that *Bt* resistance had evolved in *T. ni* populations in commercial vegetable greenhouses (Janmaat and Myers, 2003).

All sampled greenhouse populations treated with *Bt* displayed elevated levels of resistance, whereas untreated populations exhibited similar resistance levels to surrounding untreated field populations (Fig. 19.1). Grower reports of poor *Bt* efficacy corresponded to high levels of *Bt* resistance estimated in laboratory assays, suggesting that survey results were a reasonable representation of reality. Furthermore, the *Bt* resistance levels identified were directly correlated to the amount of *Bt* applied, and *Bt* resistance was observed to evolve repeatedly within 1 year as a consequence of grower spray programmes. Therefore, the survey clearly showed that *Bt* resistance was present in greenhouse *T. ni* populations and was increasing in response to *Bt* use by growers. Thus, cabbage looper

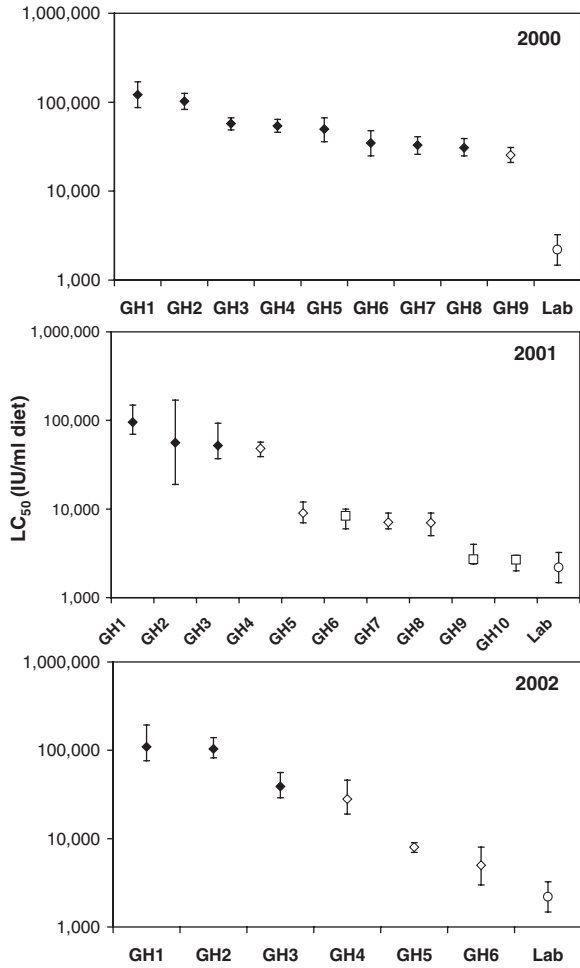


Fig. 19.1. *Bt* resistance level as shown by the *Bt* LC₅₀ and 95% confidence intervals for each *T. ni* population collected from greenhouses 2000–2002. Greenhouses in which *Bt* was applied are shown as ◆, and ◇ when untreated. Field collections are represented as □, and the reference susceptible laboratory colony is shown as ○. This figure is a modified version of Figure 1 in Janmaat and Myers (2003).

populations in British Columbia were the second reported species to have developed resistance to *Bt* outside the laboratory.

What Contributed to the Initial Evolution of *Bt* Resistance in Greenhouse *T. ni* Populations?

The very characteristics that make *Bt* a valuable microbial insecticide (i.e. no mammalian toxicity and limited environmental impact) probably also contributed to the development of *Bt* resistance in *T. ni* populations. Application rates specified on insecticide labels are developed to reduce residues on agricultural products to below certain thresholds, limit worker exposure to harmful chemicals and to reduce environmental contamination. The use of chemical insecticides above these label rates can have serious ramifications and is often monitored via residue testing.

With respect to *Bt*, residues are not problematic and *Bt* can often be applied within 24 h of harvest.

Self-reports by greenhouse growers in British Columbia suggest that applications largely in excess of *Bt* label rates were common after 2000. Therefore, the consequences of *Bt* overuse and of applying *Bt* rates above those specified were probably not felt until the development of resistance in *T. ni* populations. Since the response of a population to selection (i.e. the rate of resistance evolution) is directly proportional to the selection intensity or, in this case, the selective dose, the application of high *Bt* doses greatly intensifies rates of resistance evolution. Therefore, as *Bt* doses are increased, the time to which resistance becomes problematic for growers is shortened.

When *Bt* was first registered for use in greenhouses, growers reported that they generally applied *Bt* according to rates specified on the label. Therefore, the application of high *Bt* doses was probably a symptom of *Bt* resistance evolution and not its cause.

It is likely that the greenhouse environment played a significant role in contributing to the development of resistance. In the diamondback moth, greenhouse growing conditions have also been implicated in the development of *Bt* resistance (Tanaka and Kimura, 1991; Shelton *et al.*, 1993). Greenhouses simulate a tropical environment in temperate areas, where insect populations are protected from extreme environmental conditions, grow rapidly, undergo multiple generations per year and avoid mortality over winter. With respect to field *P. xylostella* populations, *Bt* resistance has only been detected in warm climates that allow insects to undergo multiple generations per year, such as Hawaii, Malaysia, the Philippines, Florida and Thailand (Tabashnik, 1994; Ferré and van Rie, 2002). This phenomenon is due to the relationship between the rate of resistance evolution and the number of *Bt*-treated insect generations. If an increase in the number of insect generations in greenhouses relative to field conditions coincides with an increase in the number of *Bt* applications (i.e. each generation is treated), the rate of *Bt* resistance evolution will be greatly accelerated in greenhouses. This situation is further exacerbated if resistant individuals are able to cycle between growing seasons within greenhouse structures.

Studies have shown that the overwintering ability of pink bollworm resistant to a *Bt* toxin is greatly compromised relative to susceptible individuals (Carrière *et al.*, 2001). This high overwintering mortality is thought to be a major factor contributing to the observed lack of *Bt* resistance development in pink bollworm populations in areas where transgenic *Bt* crops are grown. A similar relationship may occur in *T. ni* populations, and it is expected that resistance will decline in field populations between growing seasons; however, this decline may not occur if pest populations cycle between years, protected from inclement weather within greenhouse structures.

The favourable environmental conditions and longer growing season in greenhouses also contributes to the presence of overlapping *T. ni* life stages, which differs from the more condensed life-stage distribution generally observed in field populations. In order to control these overlapping life stages, greenhouse growers often apply *Bt* at short spray intervals to target susceptible larval stages. This practice increases the dose, owing to the addition of new *Bt* toxins to existing

residues on leaves, and the exposure period of *T. ni* larvae to *Bt*, both of which ultimately increase the selection intensity for *Bt* resistance. For example, studies of insecticide resistance demonstrate that resistance to a chemical insecticide develops most rapidly in caged insect populations when mortality caused by an insecticide is high and its persistence is lengthy (Denholm, 1983; Taylor *et al.*, 1983). Furthermore, greenhouses probably also enhance *Bt* persistence by protecting *Bt* from sunlight degradation and rain (Leong *et al.*, 1980; Behle *et al.*, 1997).

Contained environments have played a significant role in the evolution of resistance to chemical insecticides (Denholm *et al.*, 1990). Such environments reduce the influx of susceptible individuals from outside untreated populations. Immigration of these susceptible individuals is required to slow resistance development by increasing the frequency of susceptible alleles in treated populations (Georghiou and Taylor, 1986). In greenhouses, the immigration of *Bt*-susceptible *T. ni* from surrounding field populations is limited and even absent during certain periods of the year (i.e. winter). With minimal movement of *T. ni* moths into greenhouses, resistance in *T. ni* populations will develop rapidly in response to *Bt* applications, evidenced by a direct relationship between *Bt* applications and *T. ni* resistance in the greenhouse population survey.

As exemplified in this case study, microbial biological control agents are not immune to the development of resistance in pest populations. Factors shown to be significant for the evolution of resistance to chemical insecticides are equally important for microbial insecticides. The greenhouse environment can play a considerable role in the evolution of resistance to microbial insecticides owing to containment of the pest population and the protection of both the pest and pathogen from the elements. Once resistance is present, resistance problems may be exacerbated by the application of high doses. Current microbial control practices often ignore the possibility of resistance evolution, yet sustained use of bioinsecticides depends on a consideration of this phenomenon.

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20 How Much Biocontrol is Enough?

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Overview: The onion industry in New Zealand incurs severe losses from white rot disease caused by the soil-borne pathogen *Sclerotium cepivorum*. This fungus produces hardy resting structures called sclerotia, which can remain in soil for many years. A fungal agent *Trichoderma atroviride* LU132 was shown to control infection by the pathogen and formulations of it provided a 65–70% disease control. This chapter provides a personal perspective on the factors that must be considered when making the decision of how much biocontrol is enough.

Introduction

For the past decade, the Biocontrol Research Group at Lincoln University, headed by Professor Alison Stewart, has been involved in a close working relationship with Agrimm Technologies Ltd, a Christchurch-based bio-inoculant company. The partnership has 30 years combined expertise in the research, development and commercialization of *Trichoderma* biocontrol agents. Agrimm Technologies Ltd currently produce and market 15 bio-fertilizer and bio-pesticide products for use in the fruit and vegetable, turf and ornamental sectors in Australasia, the majority of recent products having arisen as a direct result of the Lincoln University (LU)/Agrimm research partnership. This chapter provides a personal perspective on the factors that must be considered when making the decision of how much biocontrol is enough.

Commercial Research Imperatives

The LU/Agrimm partnership is based on trust. However, the key contributing factor to the success of this partnership was the early recognition of the clear differences which occur between the research perspectives and the commercial perspectives and a willingness to find a suitable interface favourable to both parties.

There was an acceptance by the researchers that commercial imperatives must drive the direction of the research but, equally so, an acceptance by the business partners that the credibility of the final product would be determined by the quality and rigour of the underpinning research and development.

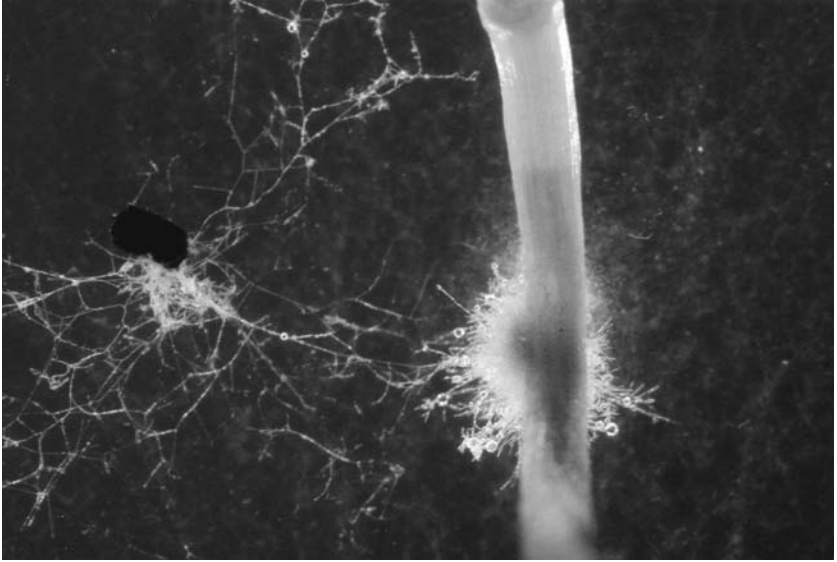
The simplistic view of how much biocontrol is enough would be to expect the biological control product to meet the same efficacy standards as those operating for chemical pesticides, which are often in the order of 80–90% disease control. However, accepting this assumption would then ignore the many other influencing factors which potentially may be more important in determining whether a biocontrol product succeeds in the market place. Setting a too rigorous benchmark could, and has, often led to missed opportunities. This point can best be illustrated by recounting personal experiences with the development of a number of *Trichoderma*-based products and, particularly, those based on the *Trichoderma atroviride* LU132 active ingredient which has formed the core of the LU/Agrim research partnership since its inception in 1995.

Solving the Onion White Rot Problem

In the early 1990s, the onion industry in New Zealand suffered severe losses from onion white rot disease caused by the soil-borne pathogen *Sclerotium cepivorum* Berk. The fungus survives in the soil as sclerotia, which are resting structures (200–500 µm in diameter) that are comprised of dense interwoven hyphae surrounded by a narrow black rind (Mordue, 1976). The sclerotia are stimulated to germinate by the onion root exudates (Fig. 20.1a), specifically the 1-propyl- and 2-propenyl (diallyl) disulphide compounds, which are the breakdown products of S-alk(en)yl-L-cysteine sulfoxide compounds exuded by onion roots (Coley-Smith and King, 1969). Once germinated, hyphae infect the onion roots or basal plate and cause cell death ahead of advancing hyphae (Stewart et al., 1989). The roots are destroyed and a semi-watery decay of the scales occurs (Fig. 20.1b) (Tims, 1948). Above ground, the leaf tips dieback and the leaves collapse (Fig. 20.1c).

The devastation caused by *S. cepivorum* in New Zealand occurred even though the growers were applying a standard spray programme of dicarboximide fungicides. Research conducted by LU showed that this loss of efficacy was due to the phenomenon of enhanced microbial degradation, where repeated applications of the dicarboximides resulted in a build-up of soil microbes able to rapidly breakdown the fungicides to less active forms (Slade et al., 1992). The onion industry subsequently invested research funds to seek new control methods. However, the industry's knowledge of biological control was limited and growers were sceptical about its promise as an alternative control strategy and, as a result, their funds were directed towards the identification of new chemicals. Although there was no support from the industry for biological control, a research project was started up with limited funds to investigate this option. Early screening work identified a number of fungal strains with *in vitro* antagonistic activity against *S. cepivorum* (Harrison and Stewart, 1988) including several *Trichoderma* isolates. *Trichoderma* species are fast-growing saprophytes that colonize a range of soil types (Gams and Bissett, 1998). This aspect, along with the antibiotic and mycoparasitic activity identified

(a)



(b)



Fig. 20.1. Onion white rot. a) *S. cepivorum* germinating sclerotium making contact with and infecting an onion root, b) infected onion bulbs, c) disease symptoms in the field. (Continued).

for a number of *Trichoderma* isolates, makes members of this genus ideal biological control agents. A series of glasshouse trials showed *T. atroviride* LU132 to be the most promising isolate, giving 50% disease control from crude formulations incorporated into the planting furrow (Kay and Stewart, 1994).

(c)



Fig. 20.1 (Continued).

Progress towards Commercial Biocontrol

More extensive glasshouse trials and small field plot trials provided evidence of levels of disease control (65–70%) considered sufficient to warrant further development of the biocontrol system (McLean and Stewart, 2000) (Fig. 20.2). A collaborative partnership was formed with Agrimm Technologies in 1995 through a government-funded ‘Biopesticides for healthy onions’ programme to develop a prototype commercial product. Agrimm formulated *T. atroviride* as a pellet, seed coat, bulk carrier and wettable powder, and 18 field trials were conducted over a 5-year period in several different cropping regions throughout New Zealand and Australia to determine the optimum formulation.

Intensive population ecology studies, utilizing a strain-specific molecular marker, showed that *T. atroviride* LU132 was rhizosphere competent and that the pellet preparation maintained fungal concentrations at 10^5 cfu/g soil compared with the other formulations, where the fungal concentration achieved was at best 10-fold less and decreased to undetectable levels over a 20-week period (McLean *et al.*, 2005). It is postulated that *T. atroviride* LU132 brings about disease control by establishing in the root region and out-competing *S. cepivorum* for nutrients and space and by producing antibiotic substances such as 6-pentyl alpha pyrone (6PAP) to inhibit *S. cepivorum* growth. The pellet formulations provided control equivalent to the standard chemical applications under low (<20%) and moderate (20–40%) disease conditions but lower efficacy under high disease pressure (>40%) (McLean *et al.*, 2002). However, industry willingness to uptake this biocontrol technology was low. This appeared to be related to their scepticism of

(a)



(b)



Fig. 20.2. Field trials showing white rot disease control given by *T. atroviride* LU132 pellets. a) untreated plot, b) treated plot.

biocontrol ('I tried a *Trichoderma* product years ago and it didn't work') and their reticence in making changes to their existing management strategies.

Such market signals did not bode well for the successful development of a commercial biocontrol product and there was strong pressure at that time to end the project. However, two events came to light over the subsequent 6 months which had a significant impact on the decision to commercialize or not. First, there was increased evidence from field trial work that the biocontrol agent had additional attributes beyond white rot control. For example, increased seedling establishment and early season growth was observed in the absence of any disease, and in the presence of disease there were yield increases observed beyond those anticipated from the level of disease control given. Even more fortunately, there were also observations from field officers that the biocontrol agent was providing some control of *Fusarium* basal rot and pink root rot (*Pyrenochaeta terrestris*). Neither of these diseases cause substantial yield losses nor warrant the application of control strategies in their own right but any control acquired during the course of other disease management practices would be advantageous to the growers.

This combination of observations added strength to the case for commercialization. The second factor which influenced the decision to commercialize was the changing market environment in the main onion importing countries. Increased market demands for produce derived from environmentally sustainable production systems forced a change in mindset within the New Zealand onion industry. Pressure to reduce pesticide use increased and the grower's willingness to consider alternative strategies followed suit. This change was promulgated even more quickly when the new groups of fungicides (e.g. triazoles) also started losing effectiveness and/or provided inconsistent control from site to site (Tyson *et al.*, 1999) and the growers felt less comfortable in relying solely on chemical control methods. Thus, the market/economic environment became favourable enough for Agrimm Technologies to move forward with commercialization and, in 2004, the product Trichopel® Ali 52 was released on a limited commercial basis (Fig. 20.3).

Successful Integration of Biocontrol

However, the story did not end there. Agrimm recognized that, under high disease pressure, the biocontrol product could not provide stand-alone control and an integrated disease management package had to be developed around the use of the biocontrol product. *T. atroviride* compatibility with pre-plant soil amendments (such as the germination stimulant diallyl disulphide, which can be used in the absence of a host crop to stimulate the *S. cepivorum* sclerotia to germinate, thereby reducing inoculum levels), fungicides and fertilizers was determined in soil pot trials and field trials (McLean *et al.*, 2001). Supported by commercial field trial results, an integrated disease management package was recommended, which combined the use of a pre-plant diallyl disulphide treatment with Trichopel® Ali 52 granules applied at planting with captan- and/or procymidone-treated onion seed, followed by late-season application of a triazole fungicide, where appropriate. This strategy maximized the opportunity for the biocontrol agent to be incorporated into mainstream onion cropping practices, reduced the number of pesticide applications and

safeguarded against the risk of 'failure' under high disease pressure. The growers could see that the biocontrol product had contributed 'added value' to their production system. Trichopel® Ali 52 also opened up the opportunity for expansion into the organic onion industry since it provided a non-chemical control measure for onion white rot, which was the main constraint to the economic production of organic onions. There is now a significant and growing commercial interest in the product for use in this integrated management strategy.

Expanding Market Opportunities

There were many lessons learned from this example. Clearly, luck played some part in the final commercialization of Trichopel® Ali 52 since the extra *Fusarium*/pink root control and increased plant growth/yield benefits was unforeseen and the inconsistent fungicide performance and changing market demands were not anticipated. However, luck often comes to those who persevere and stay committed to their goals. This became even more evident when it was discovered that *T. atroviride* LU132 had a broader spectrum of activity than first thought. Early trials had tested the isolate against a range of other soil-borne plant pathogens such as *Sclerotinia* species, *Sclerotium rolfsii* and *Rhizoctonia solani*, which are sclerotia-forming fungi that cause root and basal stem rots, with little evidence of antagonistic activity and so no further evaluations were made until more recently when, by chance, the isolate was included in some biocontrol screening work being conducted on the fruit rot pathogen *Botrytis cinerea* in strawberries. This work gave excellent results and the research was subsequently expanded by Agrimm to include evaluations against a range of *Botrytis* diseases in other crops. This has recently resulted in the New Zealand registration of a product (Sentinel®) for control of *Botrytis* grey mould in grapevines (Fig. 20.4) with additional label claims underway. Thus, the *T. atroviride* LU132 active ingredient is likely to be considerably more profitable for the commercial company than initially predicted.

Lessons to be Learned

Development of a biocontrol product does not stop when it has been commercialized. The successful launch and eventual decline of the Trichodowel®, the first biocontrol product registered and marketed in New Zealand serves as an example of the ongoing research, development and forethought required. Trichodowels are 6 mm diameter × 25 mm long sections of ribbed dowelling impregnated with a number of proprietary strains of *Trichoderma harzianum*, which act as slow-release implants when inserted into a 6 mm diameter hole drilled into the trunk of a tree. These were marketed to the orchard industry as a biocontrol agent for silver leaf disease caused by infection of pruning wounds with *Chondrostereum purpureum*, which releases a toxin that causes leaf silvering, branch die-back and often death in pip and stone fruit trees. The *T. harzianum* growing from the Trichodowel controls the silver leaf disease by acting as an antagonist to the wood-invading pathogen. Approximately 1 M units were sold between 1987 and 1995,

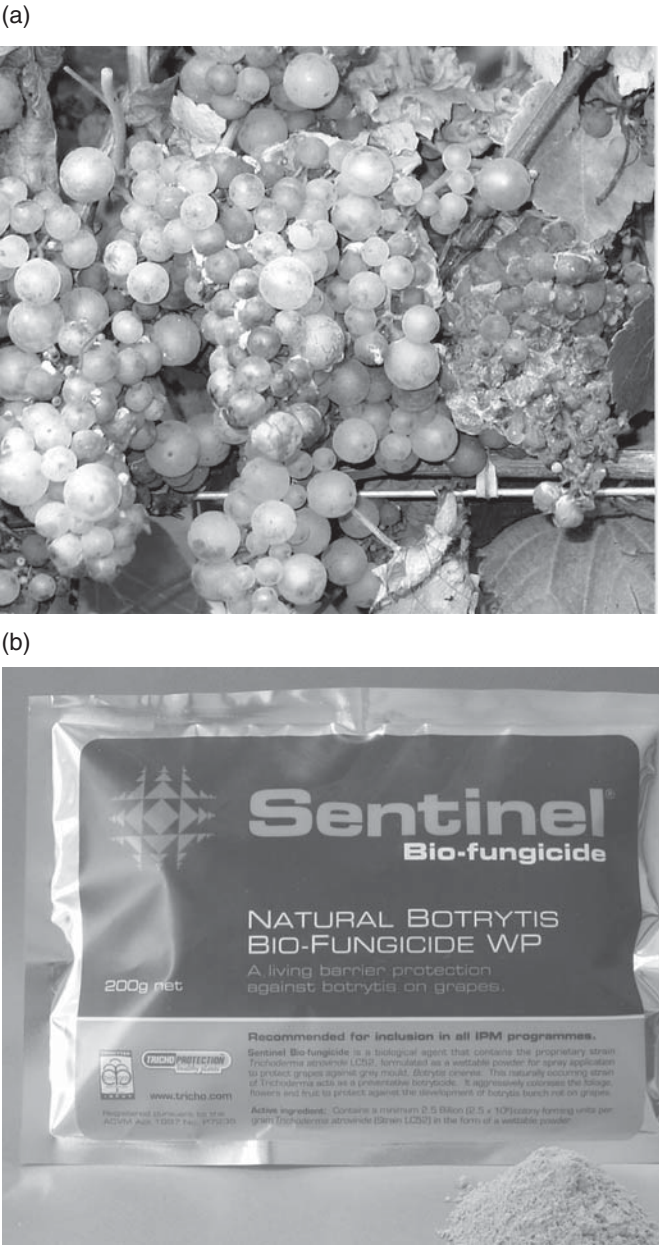


Fig. 20.4. a) *Botrytis* grey mould on grapes, b) Sentinel® wettable powder product containing *T. atroviride* LU132.

but with declining returns to the fresh fruit sector and orchards in some New Zealand areas being removed for replanting with grapevines, the market receded further and the product became commercially unviable. However, subsequent field trials in vineyards determined the Trichodowels had similar efficacy against



Fig. 20.5. a) Grapevine showing *Eutypa* die-back symptoms, b) Vinevax™ Bio-dowels.

the die-back disease caused by *Eutypa lata*, another wood-invading pathogen which causes significant loss of productivity in grapevines from the effects of leaf cupping, bunch shrivelling and eventual cordon death (Fig. 20.5a). The demonstration of protective efficacy against this disease enabled the product to be sold

into vineyards after an extension of the registration label claims. Trichodowels have since been re-branded under the Vinevax™ brand (Fig. 20.5b) and are now successfully marketed in the grape industry (Hunt, 2004).

Over the years, many lessons have been learned from past mistakes and these have been used to develop a blueprint for success. Recommendations to newcomers to the biocontrol arena are relatively simple. Be prepared to invest substantial time in evaluating the market place nationally and internationally and look at least 10 years to the future (global market trends, legislative/regulatory changes etc.). If possible, choose stable, high-value markets and biocontrol agents that can be easily produced using well-established production technologies. Assign risk factors to each of the key influencing parameters and regularly assess the status of each. Be prepared to shelve a product if the signs point to a major constraint to market success but, equally, be prepared to revisit a shelved product in light of further changes in the market situation. Plan for serendipity. Make sure that you are in a position to be able to take advantage of any good luck/unexpected opportunities that come your way. Most importantly, choose your marketing strategy very carefully. Do not market your product as a stand-alone treatment when it may be safer to recommend an integrated approach, because loss of credibility in the market place from 'over sell' of a product's capability is almost impossible to recover from.

How Much is Enough?

It is difficult to envisage that a product could be taken to the market place if it gave < 50% disease control under commercial cropping systems. A more realistic expectation would be in the order of 65% disease control. However, when the LU group identified a *Trichoderma* isolate that could provide 80% control of damping-off disease caused by *Pythium* spp. in vegetable seedlings, the system was not developed further as there were numerous chemical products (and some biological control agents) on the market which gave 95% disease control. Therefore, it is difficult to come up with a sound prediction of how much biocontrol is enough since many other factors (e.g. efficacy/cost of competing products, added premium for organic/IPM-based crop produce, additional yield or disease control benefits) come into play. It is also difficult to imagine a product being successful in the long term if it provides inconsistent control between sites or seasons. Most growers will prefer to have a product that consistently gives 75% control than one which sometimes gives 90% but at other times gives only 50%, since this is a more difficult scenario for them to manage and brings an unnecessary or unwanted risk factor into their production system.

Worldwide, there is increasing recognition and acceptance of the value of incorporating biocontrol products into crop production systems. This is particularly so in the area of high-value perennial tree crops (such as grapevines, olives, forestry, etc.), where sustaining plant vigour and health over decades is of paramount importance to economic success for the grower. Biological products with multiple attributes, such as growth promotion activity, disease suppression, minimal residue issues, etc., will become an increasingly attractive option as market demands for environmental sustainability increase. It is likely that such industries

will be prepared to accept a biocontrol product giving 10–15% less efficacy than a comparative chemical product if there is a market premium for ‘clean, green, production’. The same scenario is likely for organic broad-acre crops, where a significant premium can be obtained, but not for conventional arable crops. Over the next decade, grower education to promote the proactive use of biological products rather than the usual reactive approach used for pesticides will be essential if we are to impart a shift in mindset towards acceptance of biocontrol products as a different but immensely valuable component of pest management.

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21

Control of Root Diseases with *Trichoderma* spp. in Forest Nurseries of Central Siberia

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Overview: Chemical control of soil-borne plant diseases is not permitted in Russia. This chapter highlights factors that have influenced the acceptance and use of biological control of forest seedling production systems used in reforestation in Siberia. The chapter focuses primarily on factors that have been responsible for success or failure of biological control of forest seedlings produced in Central Siberia.

Scope of the Problem

We recognize that biological control is not a magic bullet for management of diseases that limit our efforts at production of tree nursery crops for reforestation in Russia. Our research goals were to provide additional tools for controlling such diseases. In our particular case, development of biological control was in fact a necessity, because in Russia the use of chemical fungicide is prohibited for management of plant diseases in forest nurseries. In Siberia the main cause of forest nurseries' diseases and epiphytotics are plant pathogenic fungi. Seedling mortality is often 20–30% and in unfavourable years can exceed 85% (Fig. 21.1). Siberian soils are rich in humus and have pH levels which are favourable for annual recurrence of diseases. The highest losses of coniferous seedlings are caused by damping-off by *Fusarium sporotrichioides* Sherb., *Fusarium chlamydosporum* Wollenw & Reinking, *Fusarium avenaceum* (Fr.) Sacc. and *Fusarium heterosporum* Nees. These fungi are able to survive on seed and in soil for long periods as resting spores (Gromovykh *et al.*, 2002a).

The standard disease management strategies recommended to growers include the use of fertilizers and cultural and physical practices. A few nurseries have been using a product known as 'Universal', which is derived from the commercial product Trichodermin-C, a formulation of the biocontrol agent (mycofungicide) *Trichoderma harzianum* (Samuels, 1996). This biopesticide was used for agriculture



Fig. 21.1. Damping-off of coniferous seedlings in the field.

in the western part of Russia (Gromovkyh *et al.*, 1998), where generally it provided inconsistent control at different nurseries over seasons and was totally ineffective when climatic conditions were favourable for disease development. It is not known whether this fungus was sufficiently competitive to become established in the infection zones under Siberian soil and climatic conditions.

In Russia, and likely many other countries, funding for research into biocontrol has generally been limited and short term, making it difficult to sustain a research effort. Although active groups of scientists have been working on biocontrol in Siberia, our impact on plant disease management systems has been less than what we hoped. We have, however, been able to create a significant culture collection, which was added to the All-Russian Collection of Microorganisms. Some of these strains have been patented for use as biological control agents.

Identification of Control Agents

Our investigations started in 1991 through a government programme funded by the Russian Foundation of Basic Research. The objectives were to identify new, effective biocontrol agents. A collaborative partnership with several forest nurseries of Central Siberia was started in 1992, allowing us to test new products in their fields. While we did not know how far we would get, the expectations were high. From a collection of native isolates of *Trichoderma*, 197 isolates were selected for further testing (*Trichoderma asperellum*, *Trichoderma viride*, *T. harzianum*, *Trichoderma koningii* and *Trichoderma virens*) as to their antagonistic activity against key *Fusarium* pathogens. Results from such assays identified 15 potential

strains of *T. asperellum* (Gromovykh *et al.*, 1999). Strains providing the best control under laboratory conditions were further evaluated in small field plot tests at five forest nurseries. Strain Mg-97 provided 65% disease reduction under high pressure and 85% control under moderate disease conditions. This strain was submitted to the Russian Collection of Industrial Microorganisms (F-765) and protected by a patent (Gromovykh *et al.*, 2001a, b).

Formulation

The success of biocontrol is highly dependent on the formulation process. We had already found that the form of the formulation used greatly influenced the ability of the biocontrol agent to control disease. Furthermore, the cost of the biocontrol product we manufactured was too high, primarily because the substrate used for growing the organism was expensive and the amount of biomass produced was low. We therefore set out to find a way to produce the greatest quantity of efficacious propagules in the shortest period of time. A collaborative partnership was formed with several institutes, universities and the local paper industry under a government programme 'Integration', aimed at developing biopesticide formulations (Gromovykh *et al.*, 2001a; Litovka *et al.*, 2002).

Five difficult years later we developed a formulation that we thought would meet market demands. To get here we tested solid-phase fermentation on pine bark, hydrolysis of lignin, wheat, barley and liquid fermentation on corn syrup, etc. Traditional liquid and solid technologies were not very efficient. First and foremost, few if any biopreparations have been reported to provide the high yield of spores needed for economical disease control, because of lack of oxygen during cultivation of the biocontrol agent in liquid fermentation, lack of active propagules obtained in solid fermentation, or lack of a practical or realistic approach to the study of the fermentation process. Solid-phase technology was also uneconomical because of the excessive amounts of the biopreparation needed to obtain disease control.

By good fortune we discovered a unique immersion technology for growing fungi, which combined the principles of both deep and surface cultivation. This form of fermentation was developed over 20 years at Krasnoyarsk State University for growing *Beauveria bassiana* (Tyulpanova *et al.*, 1997), a biological control agent of insects. The principle of immersive technology is immobilization of fungus on thin metal plates or strips. These strips move up and down into a liquid substrate, permitting optimal exposure times of the organism in the air yet allowing it to utilize the carbon source and increase its biomass. Once the plates are covered they are transferred to controlled environment chambers to allow for the formation of aerial mycelium and spores. This technique allows us to obtain air mycelium with excessive amounts of conidia on both sides of the metal strip.

Using this immersive technology for growing *Trichoderma* MG-97 we optimized the growth and spore production of strain *T. asperellum* MG-97 (Samuels *et al.*, 1999) and released the new product 'Trichodermin-M'. The product contains $8.5\text{--}9 \times 10^{10}$ spores and has less than 1% impurities (Fig. 21.2). The formulation is easy to apply and the spores retain viability for a long time in soil. Trials were

carried out on seedlings of key trees species grown in Siberia (*Picea obovata* L., *Larix sibirica* L. and *Pinus sylvestris* L.) at 15 forest nurseries. This allowed us to collect information as to efficacy and potential market size. From 1996–2000, plots treated with the biopesticide had between 75 and 80% healthy seedlings compared with control plots (Fig. 21.3). Application of this preparation to the seedling preparations maintained a viable fungal concentration of 10^6 cfu/g soil during the first year, and this was sufficient to prevent disease development all year. Such an extended period of protection was an important selling point for use of the biocontrol product (Gromovykh *et al.*, 2002b, 2003).

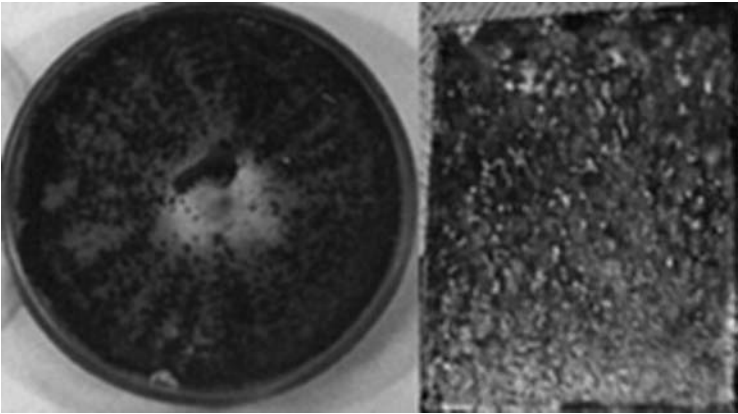


Fig. 21.2. *Trichoderma asperellum* MG-97. a) sporulating culture, b) formulated conidia of the biofungicide product.

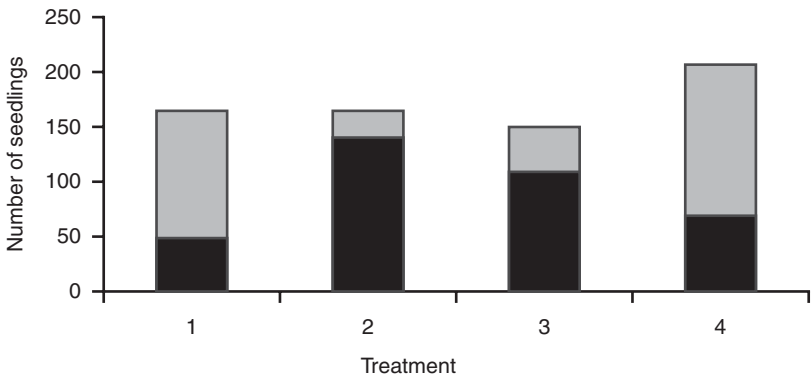


Fig. 21.3. Number of seedlings of *Picea obovata* L. per square metre after treatment with *Trichoderma* biopreparations (■ total number of seedlings; ■ number of healthy seedlings). Treatments: (1) control; (2) *Trichoderma asperellum* at 3.5×10^5 spores/m²; (3) *Trichoderma asperellum* at 2.1×10^5 spores/m²; (4) *Trichoderma harzianum* (Universal) at 3.6×10^5 spores/m².

Registration

Despite the desire by the public and growers for using low-risk methods for plant disease control, there was major concern about the risk of releasing microbes into the environment that could also have harmful side effects, even if the biological agents occurred naturally in their soils. This question had to be answered if we were to continue advocating the use of this product. The next phase of our research was dedicated to assessment of the environmental impact of biocontrol agents, particularly as to impact on microbial populations. *T. asperellum* was found to retain high activity in the community of microorganisms that it was incorporated into. It did reduce the numbers of the genera *Fusarium* by changing the structure of the microbiota. Observations by independent experts on forest production indicated that introduction of the biopesticide had an overall positive effect on mineralization of organic substances through modifications of the microbial populations. There was a decline in the number of nitrogen-fixing microorganisms but bacteria capable of using mineral forms of nitrogen increased. The populations of *Trichoderma* isolates decreased in soil after 21 days, but remained at moderate levels for 60 days after introduction. Research results indicated that the level of control of disease was consistent and satisfactory over 3 years.

In 2002 several biopesticides were registered for use on biocontrol of damping-off diseases in forest nurseries with appropriate patents for the microbial products. The registration process required the collection of data evaluating the potential hazards associated with this biopesticide. At the same time we explored the potential for commercializing the product and we are now near the end of this process. The limited market size and the large start-up costs slowed the commercialization of this biopesticide. We expect that once the product hits the market it will succeed only if companies and local authorities associated with the project help in promoting its benefits. Still, everything we know shows that our biopesticide is safer for the environment and produces healthy seedlings. We can only hope that biocontrol will have a noticeable impact on the management of large acreages of forest nurseries of Central Siberia. It has become obvious to us that the needs for disease management strategies in reforestation will never be adequately served by the amounts of money dedicated to research.

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22

Commercial Development of *Trichoderma virens* for Damping-off Disease

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Overview: *Trichoderma* species are known for their ability to control several soil-borne plant pathogens and thereby bring about control of plant diseases. In this chapter we describe how *Trichoderma virens* was developed cooperatively with industry and government into a biocontrol product GlioGard™ for management of damping-off diseases affecting the horticulture industry.

Introduction

Trichoderma species have long been known for their ability to interact with several soil-borne plant pathogens to bring about various levels of disease control (Harman and Kubicek, 1998). More specifically, *Trichoderma virens* (Miller, Giddens, Foster, and von Ark), known previously as *Gliocladium virens*, was developed cooperatively with the USDA/ARS and Grace, which registered the fungus with the US Environmental Protection Agency (EPA) for control of damping-off diseases in the horticulture industry (Lumsden *et al.*, 1996). This partnership afforded an opportunity to couple basic and applied government-sponsored research with industry expertise in product development through fermentation, formulation and marketing experience. The registration process with the EPA was a unique experience for private industry since this was the first of two fungal biocontrol products to be evaluated and registered in the USA.

Basic Requirements for the Research

Several important criteria needed to be established in the course of developing the product for commercial use. These were based on a screening method to select an appropriate microorganism for biocontrol. First, the product had to be effective in soilless growing media, where the targeted plant pathogens, *Pythium ultimum* and *Rhizoctonia solani*, caused severe losses due to pre- and post-emergence

damping-off of horticulture crops. Second, the microbes had to be native to the USA because of concerns of introducing non-indigenous species. We looked for selecting a single isolate that was active against both pathogens as this would simplify commercial production. Lastly, the target crop, and therefore the plants we used in our tests, would be of high value so that the costs of the control product could be more readily absorbed. On the basis of these criteria, over 100 fungi, bacteria and actinomycetes, mostly from areas near our station, were tested (Lumsden and Locke, 1989). Many were good candidates because of prior history of biocontrol potential. Some were promising because they were associated with dead fungal resting structures. Of all the isolates tested *Trichoderma* spp. were the best performers and one isolate, *T. virens* (GL-21), was consistently the most effective against both target pathogens.

A Product Idea Emerges

In 1982, we discussed with Grace Horticultural Products Division a problem they were having with soilless growing media in which damping-off pathogens were destroying seeds and seedlings. It was during this discussion that the idea was formulated that a cooperative would be formed between the company and our research facility to develop a biologically based soil fungicide for management of *Pythium* spp. and *R. solani*. We already had an experimental formulation for delivery and the prospective biological agents. Grace had the means to develop formulations, the fermentation capacity to grow the biological agent and the expertise to market the product through its commercial horticultural division.

The USDA had just recently implemented the mechanism to cooperate with industry and through its Technology Transfer Office a way was cleared to implement this cooperative research. On the other hand, getting the go-ahead from the company turned out to be more difficult. The Horticultural Division's upper management was fully in favour of the project, but their research arm, The Washington Research Center in Columbia, Maryland, was more reluctant to pursue this programme. After three separate written proposals and two separate verbal presentations, the upper management of W.R. Grace indicated that the project could begin as an 'unofficial' project. This meant that limited funding and time would be spent on the project. After considerable work by myself (R.L.) and Jack Lewis of the Biocontrol of Plant Diseases Laboratory, and James C. Locke of the Florist and Nursery Crops Laboratory, Beltsville, and close collaboration with two enthusiastic processing engineers, Drs James F. Walter and Jacob Eyal at the Washington Research Center, the project was awarded official project status.

Cooperative Research and Development Agreement – Jointly and Separately Agreed Upon Responsibilities

Demands to reduce the use of chemical pesticides, including fungicides, were a main impetus to develop this biological control product. At the same time, legislative

changes brought about by the passage of the US Congress Technology Transfer Act of 1986 made it possible for us to establish a Cooperative Research and Development Agreement (CRADA) between the USDA/ARS and private industry. The CRADA enabled us to work directly with staff at Grace's Maryland facility. According to the agreement, the laboratories and glasshouse facilities at Beltsville were available for basic and applied research to screen potential isolates, characterize them, develop methods to grow and formulate them, and test their efficacy against damping-off pathogens on several horticultural seed crops, carried out over the 5-year period of the agreement.

Private Industry Input

The Horticultural Crops Division of Grace managed, funded and arranged product testing with several prominent horticultural plant pathologists. In addition to supplying funding to help support the cooperative research the cooperator: (i) supplied various formulations of *Trichoderma* strains grown in their fermentation facility, for evaluation in the glasshouse; (ii) performed laboratory assays of quality control to ensure viability and potency of the formulations supplied; (iii) assisted in greenhouse evaluation of the formulations, supplied soilless potting mix and planting materials and supplies; and (iv) carried out product testing to meet registration requirements with the US EPA.

USDA Government Input

The USDA supplied personnel, equipment and facilities for the research, and in addition: (i) provided cultures of the biocontrol fungi; (ii) performed greenhouse experiments to evaluate the effectiveness of the above supplied formulations against the damping-off pathogens; and (iii) investigated the biological potential of the biocontrol strains and characterized them as to the mechanism of action against the plant pathogens.

Characteristics of *T. virens*

The *T. virens* isolate utilized in the commercial products was in our culture collection and was originally isolated from a resting structure (sclerotium) of the soil-borne plant pathogen *Sclerotinia minor* that had been buried in a local Beltsville soil. Although this was a local isolate, this species of *T. virens* is common and widely distributed throughout the world. This fungus proliferates as asexual conidia that are held in masses of moist spores. It can survive as thick-walled vegetative segments called chlamydospores, usually embedded in organic matter. The conidia spores are not airborne but are dispersed as spore suspensions in water or with soil or organic plant debris. *T. virens* is a common soil saprophyte and produces several antibiotic metabolites (Lumsden *et al.*, 1992), which are believed to enhance its competitiveness in soil. One of these metabolites, gliotoxin, is produced as the

fungus grows and develops from the formulated product added into soilless growing media. However, only minute traces of the metabolite are detectable in the product (Mintz and Walter, 1993), thus the wheat-bran-based product we developed was not considered harmful if ingested by animals.

Formulations and Delivery

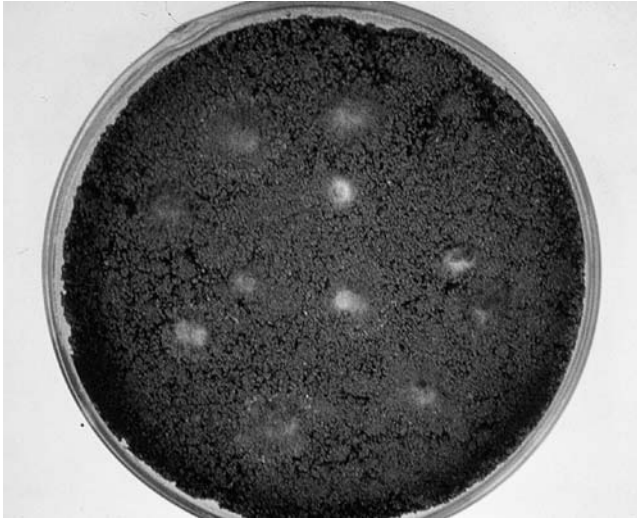
We had previously developed an appropriate formulation of *T. virens* that was easy to prepare and apply, and appeared to provide maximum protection against the two pathogens. It was based on alginate–wheat-bran granules (prill), for which US Patents No. 4,668,512; 4,724,147 and 4,818,530 were issued. The first product licensed to Grace was based upon the alginate formulation and was marketed as GlioGard™. Subsequently, certain manufacturing process scale-up problems and costs were encountered. Drying of the alginate prill was difficult in large quantities and an increase in the cost of the alginate required a modification of the product. Alginate was removed from the formulation and replaced with a less expensive carbohydrate in the dextrin group. The resulting product was formulated as a granule. This product still contained wheat-bran as a nutrient source for the fungus and was delivered to planting media in its dry granular form under the trade name SoilGard™ (Fig. 22.1). The new formulation was found to be equally effective as GlioGard but was less expensive to manufacture. Grace transferred the technology and marketing rights of the product to ThermoTrilogy Corp., and later to Certis USA, both also located in Columbia, Maryland, neither of which changed the basic formulation and marketing strategies.

EPA Registration and Safety

The US EPA requires registration of biological control agents as ‘biopesticides’. Thus, product testing is set up in a tier system that recognizes the inherent risks and degrees of exposure associated with different uses of pesticides. In addition to production and taxonomic data, long- and short-term effects on a variety of organisms including plants, animals and other non-target organisms may be necessary. Of all the possible tests that could have been required, only three were needed for registering the *Trichoderma* product. These were: (i) acute oral toxicity/pathogenicity; (ii) acute pulmonary toxicity/pathogenicity; and (iii) acute intravenous toxicity/pathogenicity.

In the animals tested, *T. virens* isolate GL-21 caused no adverse effects (Lumsden *et al.*, 1996). Three formulations were approved: WRC-GL-21, a fungal biomass product used for manufacturing; GlioGard, an end-use formulated prill product containing calcium alginate, wheat bran and proprietary additives to prolong shelf-life; and SoilGard, a granulated product with improved shelf-life that was more economical to manufacture. An environmental use permit was not required by the EPA for *T. virens* because the isolate was indigenous to the USA and because field testing was confined to small greenhouse applications. Testing for efficacy was carried out in greenhouses in locations in New Jersey, with the

(a)



(b)



Fig. 22.1. *Trichoderma virens* isolate GL-21, formulated as SoilGard™ for management of seedling diseases in greenhouse environments. a) *T. virens* growing from formulated SoilGard particles on soil, b) *Zinnia elegans* seedlings infected with *R. solani* Kuhn on left, and seedlings protected from damping-off by *R. solani* in soilless mix amended with SoilGard.

cooperation of Drs Steven Johnston and J.L. Peterson, Rutgers University, New Brunswick, New Jersey, and in North Carolina with Dr Jean B. Ristaino, N.C. State University, Raleigh, North Carolina, and in Maryland at the USDA/ARS Beltsville facility (Lumsden *et al.*, 1996).

Marketing Strategies

Successful commercialization and acceptance of the product depends on correct assessment and determination of the needs of the market and the potential for profit margins. Several issues of concern regarding this, however, were already resolved. The initial product, GlioGard, was patent protected based on the formulation.

There was a clear need in the horticultural greenhouse production system for a safe, reliable non-chemical fungicide treatment to control damping-off diseases. We had already developed a simple, inexpensive fermentation system for producing large quantities of *T. virens* in large-scale fermentors. Lastly, this biologically based disease-control product was considered cheaper to develop, register and market than a traditional chemical fungicide.

In order to assess the acceptability and market potential for a product containing *T. virens*, a test product was moved through the normal sales and distribution channels and we collected feedback from grower end-users. Visits and questionnaires were used and distribution was limited to Florida, Ohio, Michigan and Texas. From a total of 76 growers who used the product on a variety of plants and growing media, there were only two reports of less than expected performance. Most growers reported excellent disease control, uniform growth and in some cases growth enhancement.

The test marketing trial, unlike the efficacy trials, was based on grower perception. Perception determined whether or not a grower would make future purchases and whether positive information would be passed on to other growers. As expected from the results of the efficacy trials, the overall perception of the test market was very positive (Knauss, 1992).

Major Difficulties and Hurdles in the Development Process

The initial stage of convincing the management of a large corporation such as W.R. Grace & Co. that *T. virens* could be the basis of a successful biocontrol product required considerable effort. This was successfully accomplished by providing reliable data to show efficacy and the company conducting marketing inquiries to ascertain future prospects. Working closely with the Research and Development personnel at the company was essential to achieving success. At times communication between the research group and the R & D group broke down, but was rectified by appropriate meetings and communications among and between the groups. This was sometimes difficult at best because diverse skills and expertise were required from plant pathologists, microbiologists, biochemical engineers, commercial planners and administrators, and others.

Careful evaluation of quality control was of paramount importance for assuring efficacy, and efficacy determined the future confidence in the product. Three routine tests were essential to success. The first was a simple microbial quality test, which assured us that there was no external fungal or bacterial contamination of the fermentation product. This also monitored the quality of the biocontrol fungus in culture. We had available a simple, quick efficacy test for both *P. ultimum* and *R. solani* and used this routinely for evaluating efficacy of large batches of product. Random tests were performed to tested shelf-life to ensure the quality over a reasonable period of 1 year.

The product has survived several corporate transfers of ownership, which is outside the control of the developers of a product, but which shows the long-term success of a product that can withstand the vagaries of the marketplace.

Present and Future Prospects

Success in the production of more vigorous vegetable transplants through incorporation of SoilGard granules into rooting media has led to interest in application of SoilGard to field soils in order to maintain root health through transplanting and crop establishment. The current granular formulation can be incorporated into field soils for small-scale applications such as home gardens, but is less cost effective for large-scale vegetable production. One solution (also adaptable for greenhouse application) has been to prepare a slurry of granules in water, then decant the supernatant containing GL-21 chlamydospores and apply it as a soil drench to the transplant bed. Soil-borne diseases of tomatoes and peppers have also been effectively controlled by application of this supernatant via drip irrigation, although this method is still under investigation.

Development of an improved formulation that can be mixed with water for drench or drip application is currently in progress, which should allow for expanded market opportunities in agricultural crops as well as ornamental plants in greenhouse and outdoor settings. (Personal Communication, Michael B. Dimock, PhD, Director, Technical Development, CERTIS USA, 9145 Guilford Road, Columbia, Maryland 21046, USA).

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23

***Trichoderma stromaticum* for Management of Witches' Broom of Cacao in Brazil**

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Overview: Witches' broom disease, caused by the fungus *Crinipellis perniciosa*, can reduce cacao yields by 75% and is the main constraint for cacao cultivation in Brazil. In this chapter we review information about the biological control agent *Trichoderma stromaticum*, from its discovery in the Amazon basin, to its mass production and current use in the fields of Brazilian cacao farmers.

Introduction

In the Brazilian state of Bahia cacao is considered to be a low-input crop. Cultivation occurs under the shade of native tree species in a cropping system known as 'cabruca'. This environmentally friendly cropping system helps to protect the remains of the Atlantic forest, one of the most diverse and endangered biomes of the world.

Witches' broom disease, caused by the basidiomycete *Crinipellis perniciosa*, is the main constraint for cacao cultivation in Brazil (Pereira *et al.*, 1990). Since its outbreak in 1989 it has reduced yields of cacao by approximately 75% in the State of Bahia, the main Brazilian cacao-growing region (Anderbrhan *et al.*, 1999). The pathogen infects all meristematic tissues of the plant. The infected vegetative flushes develop into 'brooms', which result from hyperplasic and

hypertrophic branches formed owing to the hormonal imbalances induced by the pathogen (Fig. 23.1a). Infected flower cushions can develop vegetative brooms or parthenocarpic strawberry-shaped pods. Infected pods can have necrotic lesions on their surface and the beans inside are destroyed. The only infective propagules are basidiospores, and these are produced on the basidiocarps formed on the surface of all infected tissues (Silva *et al.*, 2002).

The disease control measures recommended by CEPLAC, the institution responsible for cacao research and extension in Brazil, include the use of copper fungicides, removal of diseased tissues, grafting susceptible trees with more resistant genotypes, and biological control as part of the integrated management of witches' broom (R.R. Valle, personal communication). Biological control is based on the application of a commercial product Tricovab, which has as its active ingredient conidiospores of the fungus *Trichoderma stromaticum*. This product has been produced since 1999 by CEPLAC (Itabuna, Bahia, Brazil; Fig. 23.1d). In this chapter we review the current knowledge on *T. stromaticum* since its discovery in the Amazon basin to its mass production and use in the field by cacao farmers.

Origin and Distribution of *T. stromaticum*

The biocontrol fungus, *T. stromaticum*, was isolated from a cacao witches' broom in Brazil's Amazon basin. It was first identified as an isolate of *Trichoderma viride* (Bastos, 1986), then later renamed *Trichoderma polysporum* (Costa *et al.*, 1996) and finally, *T. stromaticum* (Samuels *et al.*, 2000). It has proven to be the most promising biocontrol agent of *C. perniciosa*. In nature the fungus can only grow on the infected tissue (Fig. 23.1 b). A comprehensive study on the genetic diversity of *T. stromaticum* showed that two related groups exist among all isolates collected to date. Group I (GI) was found in Colombia and Bahia State, Brazil, whereas Group II (GII) occurs in the Brazilian Amazon, including the States of Pará and Rondônia, Ecuador, and Peru (De Souza *et al.*, 2006). One GII isolate was introduced by CEPLAC into Bahia State, which is located more than 2700 km away from the Amazonian region (Costa *et al.*, 1996). Thus, Bahia State is the only known place where both genetic groups occur together. Because GI isolates were never intentionally introduced into Bahia, we hypothesize that this genotype may have tagged along with the cacao plantations. Studies done by our group and CABI, Ascot, UK showed that GII isolates are found as endophytes in cacao and other closely related species such as cupuassu (*Theobroma grandiflorum*) and *Herrania* spp. Interestingly, no GI representative was ever found as an endophyte, although the surveys that have been done are not very extensive. The fungus is only found in Latin America, and always in close association with cacao, suggesting that cacao co-evolved with the pathogen *C. perniciosa* and the mycoparasite *T. stromaticum* (H.C. Evans, personal communication). It is believed that *T. stromaticum* occurs in other cacao-growing countries of Latin America and thus may be a widespread candidate for witches' broom management.



Fig. 23.1. Witches’ broom disease (caused by *Crinipellis perniciososa* and *Trichoderma stromaticum* (a mycoparasite of *C. perniciososa*). a) A hanging broom on a cacao tree, b) Apparently unimpaired sporulation of *T. stromaticum* on a witches’ broom in a plot that has clearly been treated with a copper fungicide (blue-green colour on leaf litter), c) MycoHarvester™ used to separate the spores of *T. stromaticum*, d) The commercial product Tricovab, which is packaged as shown and sold to cacao farmers.

Biology of *T. stromaticum*

Trichoderma spp. are present in substantial numbers in most soils and in environments such as decaying wood. They are the most widely used biological control agents against a wide spectrum of plant diseases. Strains of *Trichoderma* that

are effective against cacao diseases include *Trichoderma asperellum*, *Trichoderma koningiopsis*, *Trichoderma ovalisporum*, *T. stromaticum* and *Trichoderma virens*. However, *T. stromaticum* is unusual in that it can be only be found on cacao tissue infected with the witches' broom pathogen. To date it has not been isolated from soil used to produce cacao. The key to differentiate these species is available from the USDA Systematic Botany and Mycology Laboratory net site at <http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm>

Dead brooms and old infected pods are found to be colonized by *T. stromaticum*. However, this fungus is actually parasitizing the mycelia of *C. perniciosa*, and as such accelerates decomposition of the brooms, which reduces basidiocarp production and, consequently, the inoculum levels of *T. stromaticum* (Hjorth *et al.*, 2003). This mycoparasite has a certain level of specificity, colonizing and producing spores only on the surface of the brooms (Fig. 23.1b) and pods containing *C. perniciosa* mycelium (Sanogo *et al.*, 2002), although it is able to grow well on artificial media. Spores are produced on the surface of stroma, which are tightly compacted mycelial mats. Mycoparasitism is the main mode of action of *T. stromaticum* (Bastos, 1986). Other modes of action, such as induction of the plant's defence system and antibiotic production, common among species of *Trichoderma* seem not to be important for *T. stromaticum*.

Molecular studies showed that there is a considerably higher level of genetic diversity among isolates from the GII group than from the GI group (De Souza *et al.*, 2006). Isolates from GII were able to grow and sporulate at a wider temperature range and produced approximately 1000-fold more spores on rice grains than GI isolates. *Hypocrea stromatica*, a recently described species, is the sexual stage of *T. stromaticum* (Bezerra *et al.*, 2003) and it has been found in Bahia, Brazil and in Ecuador.

Mass Production and Formulation

Tricovab (Fig. 23.1d) was developed by the Biocontrol Unit of CEPLAC and has been available since 1999 for management of witches' broom disease. The active ingredient is a GII isolate originally found in Para State, Brazil. Mass production of the fungus is done on sterilized rice grains. This isolate produces approximately 1×10^7 spores/g of grain. The original product contained *T. stromaticum*-colonized rice grains with an approximate moisture content of 10%. The rice was ground up and packaged in bags containing 2 kg of Tricovab product. However, using this process the spores of *T. stromaticum* became clumped as 0.5–2 mm stromata, which did not distribute evenly in the spray droplets or blocked the nozzles and filters of the applicators. Autoclaved rice proved to be highly hygroscopic, and the high moisture content resulted in poor storage stability (Jenkins and Grzywacz, 2000). A new Tricovab formulation, made of pure spores, is now available thanks to the availability of a piece of equipment called the MycoHarvester (Fig. 23.1c) (Version 3, Imperial College Consultants, c/o IPARC, Ascot, SL5 7PY, UK; <http://www.mycoharvester.info>). This machine can break up the stromata into individual conidia and thus provides a formulation that is easy to apply (Bateman, 2004). The new Tricovab is

packed in sachets containing 40 g of pure spores. The packets are easier to handle, transport and store, and the spores provide better dosage control. The sachets do not allow moisture exchange with the environment, and this has considerably improved the shelf-life of the product. Approximately 3 kg of colonized rice are necessary to produce one 40-g sachet (Fig. 23.1d).

CEPLAC is a non-profit organization and sells the product to cocoa farmers for the cost of production, which is approximately US \$2.00 per kg of colonized rice. From 1999 to 2005, an average of 4000 kg of colonized rice was produced every year and sold to 600 farmers. It was sufficient to treat approximately 14,000 ha of cacao, from a total of approximately 600,000 ha of cacao cultivated in Bahia State. CEPLAC's Biocontrol Unit has an operating capacity to produce 4 tons of colonized rice per month. However, owing to the fact that a profit was never an objective and that many operational problems developed, the production levels were always lower than the volume demanded by cocoa farmers. Operational problems included difficulties with drying the spores and contamination with other fungi such as *Penicillium*.

Quality Assurance and Shelf-life

Particle size, extended shelf-life and proper moisture content are all aspects of quality control that need to be dealt with in commercial production of biological products. Jenkins and Grzywacz (2000) and Hong *et al.* (2001) adapted seed storage models for mycopesticide use and proved their validity using commercially available fungal parasites of insects *Metarhizium anisopliae* and *Beauveria bassiana*. Models of this type are generally useful for quantifying expected shelf-life of products and thus stock quality control. Our original product could be stored for 2 weeks at room temperature without losing viability. However, this period increased to 2 months when separation of the spores was introduced for the new product. When stored in a refrigerator, both the original product and the pure spores maintained their viability for at least 1 year. Preliminary studies indicate that reducing the moisture content of the spores to approximately 4% by exposure to silica gel for 8 h at 25°C and adding a vegetable oil improves shelf-life even further. These improvements will be incorporated into a new formulation of the product in the near future.

Field Experiments and Applications

CEPLAC recommends applying Tricovab four times, at monthly intervals from May to August, for the management of the witches' broom disease (R.R. Valle, personal communication). This corresponds to the rainy season in the region. The effective concentration to be applied is 10^{11} conidia/ha sprayed in 300 l of water. One 40-g sachet contains a sufficient amount of spores for treating 1 ha and the treatment costs approximately US\$6.00. Efficacy is improved when precautions are taken to ensure that the fungus can actively colonize the dead brooms. Thus one must consider that the biological control agent is sensitive to

UV radiation and drying. For applications during the dry season, an agricultural emulsifiable vegetable oil and a carbon source such as sucrose can be added to the suspension, both at a concentration of 2%. Adding the vegetable oil to the pre-mixture in water helps improve the homogeneity of the spore suspension. Tricovab can be sprayed with a manual backpack sprayer; however, brooms at the top of the tree canopy can be reached more efficiently by using a motorized sprayer.

We also recommend that Tricovab be sprayed over the brooms on the cacao crop litter, where the antagonist reduces basidiocarp formation by up to 99% (Costa *et al.*, 1996). Cacao fields treated with *T. stromaticum* had a significant reduction in the inoculum of *C. pernicioso*, as measured by counting the number of basidiospores collected on spore traps, when compared with a non-treated area (K. Roncato and G. Leal, unpublished). Depending on the environmental conditions, *T. stromaticum* can reduce the number of basidiocarps formed on suspended brooms by 89% (Hjorth *et al.*, 2003). Following four sprays of the product in another trial, brooms colonized by *T. stromaticum* increased from 0 to 63% in treated plots. In addition, results from a 2-year field trial showed that Tricovab increased cocoa yield by 30%.

T. stromaticum can be sprayed as a tank mixture with most of the fungicides, insecticides and herbicides used on plantations. Among these products, the herbicide glyphosate and copper-based fungicides (copper hydroxide, cuprous oxide and cuprous oxychloride) are the most used on cacao and are not harmful to *T. stromaticum* (Fig. 23.1b). Only the fungicide tebuconazole and the combination of herbicides paraquat + diuron are not recommended for combined use since the number of brooms colonized by *T. stromaticum* was reduced by 85% and 90%, respectively, by these treatments.

Field trials done in Peru demonstrated that *T. stromaticum* reduced the number of pods infected with *C. pernicioso* by 48% (Krauss and Soberanis, 2002). However, its efficacy was more pronounced in areas with a relatively lower disease severity. Unfortunately the fungus did not affect black pod and frosty pod rot, caused by *Phytophthora* spp. and *Moniliophthora roreri*, respectively, confirming its specificity to *C. pernicioso*. In Ecuador, where *T. stromaticum* is indigenous, the fungus has been mass produced and is currently being evaluated under field conditions for controlling witches' broom disease (C. Suarez, personal communication).

Although no official survey has been done, most farmers appear to be satisfied with the performance of Tricovab. When asked about the effect of the treatment in the field, farmers usually answer that *C. pernicioso* inoculum levels have decreased and pod production has increased since the biological product was applied. Another positive effect seen is the acceleration of witches' broom decomposition, which may result in reduction of inoculum production.

Outlook

T. stromaticum is being studied by CEPLAC, Almirante Cacao, UESC, USDA, and other national and international research institutes. The objectives of these

research programmes are comprehensive, such as to study the physiology, diversity and environmental impact of *T. stromaticum*, and obtain more efficient isolates with respect to sporulation, dissemination in the field and virulence to *C. perniciosa*. Better formulation and application technologies are the most needed areas in relation to *T. stromaticum*. The ideal situation would be the application of *T. stromaticum* on the brooms in the canopy to achieve what could be called 'biological broom pruning', avoiding the need to remove dead brooms, which is one of the most expensive practices adopted on cocoa farms for controlling witches' broom. Current indications are that *T. stromaticum*, with its ability to specifically parasitize the witches' broom pathogen and to spread naturally, could be included in the category of a 'classical biocontrol' agent (H.C. Evans, personal communication). To the best of our knowledge this is the first time that a tropical tree crop pathogen was being managed by introducing its natural parasite into an agricultural area. Increasing or creating 'hotspots' of the biocontrol agent would help in its long-term establishment in cacao farms currently infected with the witches' broom pathogen.

Large-scale production by commercial companies could improve the availability and total area treated with Tricovab by farmers. The current production levels are obviously inadequate. Discussions are in progress with private companies in Brazil for registration and scale-up for commercialization of Tricovab. There is a potential for Tricovab to be sold in countries in addition to Brazil with the witches' broom problem, such as Ecuador, Colombia, Venezuela, Trinidad, Peru and Bolivia. It may also have a role in preventing the spread of the disease to other cacao-producing countries where the disease does not currently exist. Environmentally friendly characteristics of *T. stromaticum*, such as being non-pathogenic to humans, animals and plants, its ability to spread naturally, and the apparent ready acceptance of Tricovab by farmers, make the further development of *T. stromaticum* for biocontrol of witches' broom a priority.

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24

Lessons Learned from *Sporidesmium*, a Fungal Agent for Control of Sclerotia-forming Fungal Pathogens

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Overview: Soil-inhabiting fungi such as *Sclerotinia minor* and *Sclerotinia sclerotiorum* attack a wide variety of crops, causing severe economic problems. The pathogens survive for many years in soil by forming black, seed-like resting structures called sclerotia. These are very difficult to eradicate once they have infested a soil. This chapter details how the thorough understanding of the life cycle and ecology of the fungal pathogen allowed for discovery and implementation of a fungal hyperparasite, *Sporidesmium sclerotivorum*, into a disease control strategy for lettuce production.

Introduction

Given what we knew and how we approached biocontrol of soil-borne plant pathogens in the late 1970s, most plant pathologists probably would not have discovered *Sporidesmium sclerotivorum* for use as a biocontrol agent. While this fungus still has lessons to teach us about epidemiology (Gubbins and Gilligan, 1997; Stolk *et al.*, 1998; Jeger *et al.*, 2004) and control of other diseases (del Rio *et al.*, 2002), this chapter attempts to provide insight into some of the thought processes and logic behind the initial discovery and use of *S. sclerotivorum*.

The Importance of Fieldwork

This story begins in the 1970s when Peter Adams began searching for a biocontrol for management of lettuce drop, caused by the soil-inhabiting fungi *Sclerotinia minor* and *Sclerotinia sclerotiorum*. These soil-inhabiting fungi attack a wide variety of vegetable and other crops, causing severe economic problems on many hosts.

The disease is difficult and expensive to manage, requiring multiple sprays of fungicides. The pathogen survives for 3–8 years in soil as sclerotia.

To study the disease under production conditions, Peter Adams planted lettuce in the field and inoculated plants with *S. minor*. He hoped to increase the pathogen and disease by successively cropping lettuce, in order to have a disease nursery in which to conduct experiments. Instead of more disease in successive croppings, there was less disease. Thus, the choice arose whether to try again in a new location or to understand why there was less disease rather than more over time. Given that disease incidence lessened even in the presence of a susceptible host and a favourable physical environment, the possibility of a native biocontrol agent(s) at this site seemed worth investigating.

Think About What Kind of Antagonist and Design Your Screening Process to Find What You Are Looking For

At this time in plant pathology, searching for new biocontrol agents against soil-borne plant pathogens usually meant isolating candidate microbes from soil, screening them by placing them in dual culture with the pathogen or determining the extent to which they were rhizosphere competent. Peter Adams chose instead to bury sclerotia of the pathogen in the field, retrieve them, and plate them not only on agar, but also on moist filter paper. After 2 weeks, conidia of a new species of *Sporidesmium*, *S. sclerotivorum*, formed on sclerotia and on hyphae that had grown out on to the filter paper (Uecker *et al.*, 1978). *S. sclerotivorum* grows very slowly and is easily outgrown by other microbes. Further, it is fastidious and cannot be cultured on standard media. Thus, plating the sclerotia on filter paper was a wise choice.

Information on the Basic Biology and Epidemiology of the Antagonist Can Provide Insight about What Organisms Might Be Exploited for Biocontrol

Studies on the basic biology of *S. sclerotivorum* indicated that in nature it behaves as an obligate parasite on sclerotia of *S. sclerotiorum*, *S. minor*, *Sclerotium cepivorum* and *Botrytis cinerea* (Ayers and Adams, 1981). Conidia of *S. sclerotivorum* germinate in response to the presence of host sclerotia and can detect these sclerotia at distances of up to 9 cm (Ayers and Adams, 1979). Mycelium of *S. sclerotivorum* from an infected sclerotium can grow up to 3 cm in soil to infect new sclerotia (Adams, 1990). Hyphae from germinated sclerotia penetrate the intercellular matrix of the sclerotia, forming haustoria in the cells of the sclerotia (Bullock *et al.*, 1986). Each sclerotium of *S. minor* supports production of approximately 15,000 conidia of *S. sclerotivorum* (Adams *et al.*, 1984).

Knowledge of the Host Plant, Pathogen and Cropping System Reveals Where and When Biocontrol is Most Likely to Work

The pathogen overwinters as sclerotia in soil. Infection of lettuce by *S. minor* and *S. sclerotiorum* can occur in several ways. The sclerotia can germinate to form hyphae, which then penetrate into the herbaceous stems and leaves near the soil. In the spring, *S. sclerotiorum* produces apothecia, from which ascospores are ejected and become wind-borne. These ascospores can also cause infection. Disease symptoms include water-soaked lesions, often accompanied by tufts of fluffy, white mycelium. On lettuce, lesion formation is followed quickly by wilting and the complete collapse of the above-ground portion of the plant. New sclerotia are formed in and on the dead plant tissue. This fairly simple observation proved to be the essential element to the successful implementation of this biocontrol agent. Biocontrol against soil-borne plant pathogens had been generally ineffective as the pathogens are distributed throughout the soil mass and therefore difficult to locate and target. A hectare of soil to 15 cm depth weighs about 2 million kg and hitting a small target such as a sclerotium is next to impossible. Researchers thus relied on two common strategies for biocontrol of soil-borne pathogens, namely: protection of the infection court by introducing the control agents to the root zone (think rhizosphere competent), and slowing reinvasion of a pathogen into soil that has been previously disinfested by a chemical or physical sterilization procedure. The discovery that in the lettuce–*S. minor* system some of the pathogen propagules are exposed on the surface of the decayed plant tissues and can be readily targeted for treatment with a biological antagonist provided a window for control of this organism not previously experienced.

On romaine lettuce, an average of 3450 sclerotia of *S. minor* are formed per plant and as many as 12,287 were recovered from a single plant (Adams, 1986). Further, because of their original association with the plant, the sclerotia are in an aggregate distribution and they maintain this aggregate distribution even with subsequent disking of the field (Adams, 1986). The aggregate distribution of the pathogen is another simple, but key, factor in success.

Think Holistically; Think Ecologically

Astute and biologically minded readers will by now have put the pieces of these various systems together. At the end of the growing season, marketable lettuce is harvested. The crop residue, with some sclerotia exposed on its surface, is sprayed with conidia of *S. sclerotivorum* and the residue is ploughed or rototilled into the soil. The mycoparasite parasitizes a sclerotium and then grows into neighbouring sclerotia to parasitize them. The population of *S. sclerotivorum* increases in direct relation to the population density of sclerotia of the pathogen, as in a predator–prey relationship (Adams *et al.*, 1984). It is important to know that the only inoculum produced by *S. minor* is the overwintering stage, the sclerotium. Thus, destruction

of the overwintering stage is a very effective strategy for management of this pathogen.

It Has to Work in the Field

In spite of all the elegant work we might do on mechanisms and other basic aspects of biocontrol, the only factors that most people actually care about are to what extent and how consistently disease can be managed in the field using this biocontrol organism. Thus, a multi-year field test was set up. Before setting out the field test, soil was sampled to find a location where *S. sclerotivorum* could not be detected. A site planted in well-established turf was selected. Plots were 3 × 3 m with 3 m of undisturbed turf between plots (Adams and Fravel, 1990). In the autumn, lettuce was planted and inoculated with *S. minor*. When disease was fully developed, plants were rototilled into the soil. The following spring, soil was assayed to determine population sizes of the pathogen and lettuce was again planted. After disease had developed, plants were sprayed with conidia of *S. sclerotivorum* at rates equivalent to 0, 0.2, 2 or 20 kg/ha. Immediately after application, plants were rototilled into the soil and plots were sprinkler irrigated. There were no additional applications of the biocontrol agent in subsequent seasons. Lettuce was planted in these plots for the next five seasons (autumn and spring). The location and date of first symptoms of each lettuce plant was recorded. At harvest, apparently healthy plants were harvested and all other plant material was rototilled into the soil. Populations of the pathogen and mycoparasite were monitored. By the end of the fifth season, *S. sclerotivorum* had eradicated or nearly eradicated the pathogen from all treatments, including the control treatment (see below). Disease in autumn crops always began later after planting than in spring crops, and disease progressed more slowly in the autumn (Fravel *et al.*, 1992). When inoculum doses of *S. sclerotivorum* were increased, the incremental levels of disease reductions became smaller and smaller. We therefore concluded that the optimal rate for application of the mycoparasite was just under 0.2 kg/ha.

Give Fungi the Opportunity to Do What They Are Genetically Programmed to Do

As indicated above, we showed by soil tests prior to the experiment that *S. sclerotivorum* was not present in the test plots. To prevent cross contamination of treated versus control plots we maintained 3 m wide grass strips between plots and the work was always done in control plots before treatment plots. Nevertheless, between the second and third season of the trials, *S. sclerotivorum* was detected in the control plots, indicating that either it was present prior to initiation of the experiment in populations below the detection limit or, despite our best efforts, control plots became contaminated by treatment plots (Adams and Fravel, 1990). In either case, the population of *S. sclerotivorum* in control plots increased from a trace amount to levels that eventually killed nearly all sclerotia. We were therefore left with control plots that also had virtually no disease.

Problems Are Sometimes (But Not Always) Advantages

S. sclerotivorum is very restricted in the types of sclerotial fungi it attacks, and therefore, from a commercial point of view, offers a limited market. While this could be considered a disadvantage, we consider it an advantage, since there are no known non-target effects. A second complication as to commercialization was that *S. sclerotivorum* is a biotroph and is difficult to culture, although media for growing it have been developed (Ayers and Adams, 1983; Mathew and del Rio, 2004). Furthermore, *S. sclerotivorum* is very slow to act and is unlikely to provide control in the season in which it is applied. Indeed, *S. sclerotivorum* is best applied at the end of the growing season. While the disadvantage of the need for advanced planning is not likely to change, the compatibility of *S. sclerotivorum* with several fungicides (Adams and Wong, 1991) should permit integration of *S. sclerotivorum* with crop(s) being grown while *S. sclerotivorum* is parasitizing sclerotia. Because control can be achieved with such a small amount of inoculum and a single application of *S. sclerotivorum* provides control for more than one season (Fravel *et al.*, 1992), control using *S. sclerotivorum* is more economical than multiple applications of fungicides (Adams and Fravel, 1990).

There Is More Than One Way to Solve a Problem

The example of *S. sclerotivorum* is somewhat of an anomaly in the plant pathology biocontrol literature. However, the approach is appropriate for this particular combination of plant, cropping system, pathogen and biocontrol agent. Exploitation of features of each of these components allows for multi-season management of lettuce drop with a single application of only 0.2 kg/ha of *S. sclerotivorum*. Of particular importance is that we have used to our advantage information as to the accessibility of sclerotia formed above ground, the fact that sclerotia are aggregated and that this increases the rate of their destruction by *S. sclerotivorum*. Equally important was the realization that while others were successful in biocontrol of soil-borne plant pathogens with strategies of root colonization by biocontrol agents or slowing movement of pathogens through soil, different approaches could be equally valid.

In summary, *S. sclerotivorum* reinforces what our best science teachers were trying to tell us. Our minds should always remain open for chance observations that lead us to unexpected answers.

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25 Sporodex[®], Fungal Biocontrol for Powdery Mildew in Greenhouse Crops

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Overview: Powdery mildews can cause severe losses to crops in both field and greenhouse conditions. A yeast-like fungus, *Sporothrix flocculosa*, was isolated from powdery mildew infection sites and found to be an exceptional control agent of powdery mildew on a number of crop plants. This chapter describes the research effort and the trials and tribulations of bringing this product to market under the trade name Sporodex[®].

History of the Biological Approach to Powdery Mildew Suppression

Although there were many references to biological control prior to 1960, it was perhaps the lunchtime discussions at Berkeley in 1963–1964 that crystallized concepts of biological control in the minds of such luminaries as W.C. Snyder, K.F. Baker, C.E. Yarwood, T.A. Tousson, Z.A. Patrick and R.J. Cook.

Early biocontrol in greenhouse crops

An opportune climate for development of biological controls for greenhouse crop diseases came about by the coincidence of a number of circumstances. First, in the early 1970s, there was increasing concern with the problems posed by the tolerance of many fungal plant pathogens to fungicides. The response of many growers was to increase the dose rates as well as the frequency of applications, a losing battle that often resulted in yield and quality losses. In addition, consumers were becoming increasingly concerned with the visible and invisible residues

on produce. Later that decade, growers began to use computers to monitor and control the crop and the greenhouse environment more rigorously than previously possible. At about the same time, biological control of insects became more integrated into production, and growers became concerned about the effects of chemicals on the predatory insects that were used for pest management. The most important development, however, was the introduction of bumblebees for pollination in greenhouse vegetable crops. Bumblebees were found to be much more effective pollinators than the manual techniques previously used and the yield increases they brought about changed the economics of the industry. However, they turned out to be extremely sensitive to many types of pesticides, and growers were forced to switch away from chemicals as much as possible.

First efforts against diseases

In the late 1970s, the first author was focused on battling a new *Fusarium*-incited disease of tomatoes, for which no fungicides were effective. This disease threatened to wipe out this industry. It became apparent that the disease severity was actually linked to the practice of soil sterilization with fumigants, as killing soil microorganisms created a biological vacuum. We examined the potential of introducing microbial competitors to this newly described pest, *Fusarium oxysporum* f.sp. *radicis-lycopersici*. To accomplish this we added green manure to freshly sterilized soil. Of the amendments tried, lettuce and dandelions were the most effective, either grown as companion crops or by incorporation of their residues prior to the planting of tomatoes (Jarvis and Thorpe, 1981). Subsequent work showed that it was not biological control that was reducing disease but rather the iron chelation by caffeic acid and its o-diphenol derivatives in these crop residues that probably inhibited fungal activity (Kasenberg and Traquair, 1988). Such an allelopathic control needed no registration at that time.

In a similar approach, we searched for an organic amendment that would enhance the activity of the well-known biocontrol agent, *Ampelomyces quisqualis*, a common hyperparasite of powdery mildews. Various nutrients (sugars, peptone, etc.), combined with glycerol to maintain the moist microenvironment the fungus requires, were used to encourage the activity of this fungus. The nutrient sprays often enhanced control of *Podosphaera xanthii* (syn: *Sphaerotheca fuliginea*) on cucumber leaves. However, control was not always by the fortuitous arrival of *A. quisqualis* but rather by the presence of a yeast-like fungus (Jarvis, 1992). Unfortunately, this isolate was lost following the closure of the Glasshouse Crops Research Institute (GCRI) in England.

Regardless of the microorganisms involved, it was then argued to the registration authority that foliar nutrient sprays were not fungicides but foliar fertilizers, which did not require registration. That argument failed and 'foliar fertilizer' work was abandoned. The recognition of yeast applications as foliar fertilizers is not a viable approach for expeditious registration of biocontrol agents because claims for disease control cannot be made for registered fertilizers. Curiously, it might be noted that although *A. quisqualis* was commercialized as AQ10, it has

not been very successful for greenhouse work, probably because of the rather exacting wet conditions it needs for infection of the powdery mildew.

Isolation of new antagonists against powdery mildew

Notwithstanding, increasing problems with chemical fungicides in greenhouse crops in the mid- to late-1980s stimulated a renewed search for a yeast-like fungus on Ontario powdery mildews in the hope of finding a similar fungus to the one lost at GCRI. Using a standard spore-drop technique for leaf disk samples of field plants (such as red clover and dandelions) infected with powdery mildew, several dozen commensal fungi associated with mildewed leaves were isolated (Jarvis *et al.*, 1989). Using moist Petri dish chambers containing cucumber leaf disks infested with powdery mildew, two new yeast-like fungi, *Sporothrix flocculosa* and *Sporothrix rugulosa*, were identified as being highly effective for reducing the infection of cucumber by powdery mildew (Jarvis *et al.*, 1989). Their taxonomic identity was subsequently linked in 1995 to the anamorphic basidiomycetous yeast *Pseudozyma* using DNA sequencing techniques (Boekhout, 1995). These fungi are not internal parasites of the powdery mildew thallus, as is *A. quisqualis*, but they secrete a number of antifungal compounds (Benyagoub *et al.*, 1966; Choudhury *et al.*, 1994; Avis *et al.*, 2000; Cheng *et al.*, 2003) that very quickly kill the mildew agent (within 24–48 h) under appropriate, but not too exacting, environmental conditions (i.e. relative humidities greater than 70%) (Jarvis *et al.*, 1989).

On the road to biofungicide development

In 1989, the Biocontrol Laboratory at Laval University (under the leadership of R.B.) initiated a research project on powdery mildew biocontrol in which several fungal antagonists, including *Pseudozyma flocculosa* and *Pseudozyma rugulosa*, were evaluated for control of powdery mildews of greenhouse crops. Results highlighted the consistent superiority of *P. flocculosa* as a biocontrol agent of powdery mildews. Based on these results, Plant Products Co. Ltd agreed to invest in the development of *P. flocculosa* as a biofungicide through the University–Industry partnership programme of the Natural Sciences and Engineering Research Council of Canada (NSERC). Even though it was feared that too much data had already appeared in the scientific and technical literature, a patent, as well as registration, for this biological control process were sought. These quests have taken 15 years, with seemingly as much time spent with lawyers and registration officials as in furthering science and technology transfer.

In the course of the project, studies of efficacy, host–parasite relations, mechanisms of disease suppression, compatibility with greenhouse pesticides and mass production were conducted at Laval University and at AAFC Research Centers in Harrow and London, Ontario (Hajlaoui *et al.*, 1992, 1994; Bélanger *et al.*, 2002).

Mass production and formulation

While the research aspects of the project were carried out at a normal pace, our initial optimism at 'marketing' a biofungicide within a few years was quickly shattered by product development and the seemingly endless data requirements for registration. At the centre of the challenge were issues surrounding mass production and formulation. Our numerous efforts to find a Canadian (or American) collaborator for mass production and formulation were unsuccessful for a number of reasons, including lack of facilities, lack of expertise and high costs of fermentation units. Considering that development of a commercial formulation was essential to fulfil toxicology tests for registration, tests which in turn were essential to obtain a research permit to carry out commercial trials, trials which in turn required large amounts of formulated biomass, which in turn required large amounts of money that any industrial partner would wisely not invest unless successful commercial-scale trial data was available, we were soon caught in what appeared to be an inextricable catch-22 situation. The launching of a new collaborative R&D programme by the Quebec Government, the Synergie Program, gave new life to the project by injecting monies into the development of a commercial formulation of *P. flocculosa* known as Sporodex®.

The bulk of the research effort toward mass production and formulation took place in Belgium with a partner that specialized in microbial products. Countless experiments were carried out to optimize fermentation conditions as well as formulation of the extracted biomass. The number of parameters that had to be monitored for each batch was sometimes overwhelming (e.g. spore concentration, spore survival over time, presence of contaminants (at least five different tests for each batch), storage conditions, efficacy, survival during shipping, cost, etc.). While our Belgian collaborators were very helpful, overseeing the experiments from overseas, maintaining research staff on the premises and getting access to the fermentation units proved to be very difficult.

Commercial trials

At the same time, coordinating commercial efficacy trials with an adequate supply of Sporodex® (the quality having to meet registration standards) was very challenging. Nevertheless, several trials were carried out under semi-commercial and commercial conditions of greenhouse cucumber and rose production in Ontario, Quebec, the USA, the Netherlands, France, Greece and even Colombia (Bélanger *et al.*, 1994, 2002; Dik *et al.*, 1998). Most of these studies showed that Sporodex® performed at least as well as (if not better than) the best recommended fungicides (such as dodemorph-acetate and microfine sulphur) (Bélanger *et al.*, 2002) and the biocontrol agents AQ10 and *Verticillium lecanii*, even sometimes giving an increase in yield of cucumbers without evident residues (Bélanger *et al.*, 2002). Similar results were also obtained in controlling rose powdery mildew (*Podosphaera pannosa* var. *rosae*) in Québec and on other crops in the Netherlands (Bélanger *et al.*, 1994, 2002; Dik *et al.*, 1998). In addition to disease suppression, improved flower quality in some cultivars of roses was associated with the elimination of

stress and the phytotoxicity imposed by fungicide applications in the greenhouse (Bélanger *et al.*, 2002). The biocontrol agent was also found to be compatible with other pest control products and is potentially useful in integrated pest management systems for greenhouse crops.

Mode of action

The antifungal activity of *Pseudozyma* saturated fatty acids (Benyagoub *et al.*, 1966; Choudhury *et al.*, 1994; Avis *et al.*, 2000) and glycolipid surfactant (flocculosin) (Cheng *et al.*, 2003) on membranes of conidia of powdery mildew and other fungi has been confirmed by chemical studies and insertional mutagenesis that knocked out the ability of *Pseudozyma* to produce these antifungal compounds (Bélanger *et al.*, 2002; Cheng *et al.*, 2003; Mimee *et al.*, 2005). Naturally occurring resistance to these compounds has not been detected and its development in the pathogen is highly unlikely in view of the instability of the antifungal compounds in nature. Molecular markers were also developed for much-needed ecological studies on the spread and survival of this yeast-like biocontrol agent within and outside the greenhouse environment (Fig. 25.1) (Caron *et al.*, 2005; Neveu *et al.*, 2006).

Frustrations and pitfalls that could have been avoided

Patent

Hindsight is 20/20 vision. Many of our problems and frustrations could have been avoided. Early patent protection is vital in attracting the interest from the pest control industry in commercializing a microbial biocontrol product. Had we been

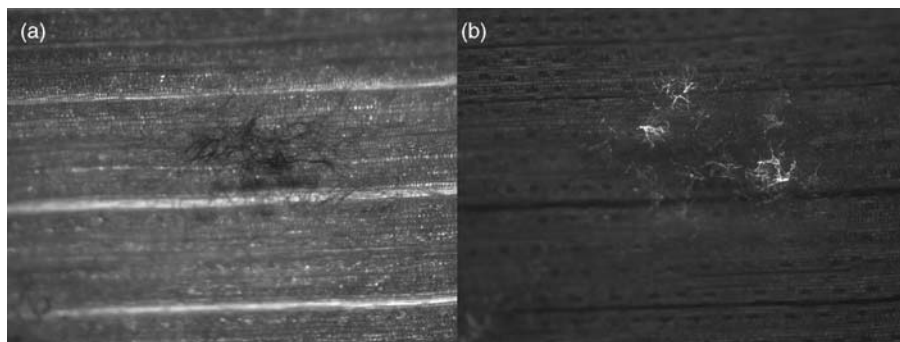


Fig. 25.1. Microscope observations of the interaction between *P. flocculosa* Act-4 and the powdery mildew pathogen *Blumeria graminis* f.sp. *tritici* on wheat leaves. *P. flocculosa* Act-4 is a strain that has been transformed with green fluorescent protein. Notice that fluorescence on the right is concentrated nearly exclusively in areas where powdery mildew colonies were present. All observations were made at 25x under natural light (a) or blue light (b).

aware that any disclosures, i.e. research papers or published proceedings and abstracts at conferences, would jeopardize patent approval, we would have been more secretive at the time of discovery in 1988 and 1989. At that time, exchange of scientific information and publication were the main objectives of government and university researchers. Now, we know that European patent offices disallow any disclosures, while the Canadian and US patent offices allow a grace period of 1 year after publication. There still can be a long waiting period between submission and patent approval. A Canadian patent was granted in 2000 for the biological process of mildew control entitled 'Methods and composition for the biological control of plant diseases' (Canadian Patent Application No. 2,011.705, originally filed in 1990).

Registration

Registration of Sporodex® as a biological fungicide in Canada was granted by the Pest Management Regulatory Agency (PMRA) in 2002 and in the USA by the Environmental Protection Agency (EPA) in 2003. Some of the frustrations and delays that we experienced in registration were caused by the lack of guidelines for the registration of microbial disease control products in Canada. These guidelines are now available from PMRA administered by Health Canada and have been harmonized with regulations by EPA in the USA. In fact, Sporodex® was the case study in the development of these regulations in Canada. Close contact and early pre-submission consultation with PMRA is the best way for researchers and biocontrol companies to avoid delays and frustrations in the registration of microbial products.

Screening potential agents

Isolation and dual-culture screening *in vitro* of potential antagonists of disease-causing fungi are only the beginnings in the discovery and development of biocontrols. Hundreds of isolates and strains can be amassed and it is important to develop a realistic and efficient bioassay for efficacy in suppressing disease symptoms on plant material. We were lucky to have developed a rapid, moist-chamber bioassay of yeast-like mildew antagonists on rose and cucumber leaf disks early in the discovery process. Successful mildew control candidates were then screened on whole plants in humid greenhouse environments. Environmental conditions for optimizing disease suppression under semi-commercial and commercial greenhouse conditions were determined early in the development of the biological control scheme.

Identification of potential agents

Accurate and precise identification and naming of the active microbial agents are also very important aspects of the research because these activities can influence the smooth progress of registration. For example, some of our early frustrations were due to the incorrect identification of the antagonistic yeast based on microscopic

morphological characters. Identification of the fungus as an Ascomycete with a *Sporothrix* anamorph ignited warning flares and health cautions because of a possible taxonomic relationship between our yeast-like biocontrol agent and *Sporothrix schenckii*, which is a troublesome dermatophyte from peat moss. These clinical fears were allayed finally by molecular taxonomists, who showed that the yeast-like fungus was actually an anamorphic Basidiomycete aligned to Ustilaginales.

Toxicity issues

The other concern focused on the mechanism of disease suppression and our use of the term 'antibiotic' to describe the antifungal activity of the natural products produced by our antagonist. Antibiotic resistance is a major health and environmental concern. We were able to show that the antifungal compounds produced by Sporodex® are very labile and are not expected to be a health and safety concern in nature. However, there is a fear that disclosure of antibiotic production will hinder the registration process for biocontrol agents, and some scientists will purposely avoid investigating this mode of action for fear of jeopardizing registration.

Ecological studies of the biological control process on plant surfaces, particularly the physical conditions that enhance growth of the microbial antagonist, are necessary for assurance of sustainable efficacy in disease suppression in commercial greenhouses. Microecological studies in the rose and cucumber phylloplane have been frustrating because the instruments and technologies for precise study of microbial function and microbial interactions on the leaf surface were lacking. *P. flocculosa* is most effective after 48 h at vapour pressure deficits below 0.6 kPa and temperatures between 22°C and 30°C (Jarvis *et al.*, 1989). The modern greenhouse industry relies heavily on accurate computer control of the crop and its macroenvironment within the greenhouse (Clarke *et al.*, 1994), so environments conducive to successful biological control with products like Sporodex® now ought to be achieved with greater ease. However, little practical attention has been paid to the management of the environment within the boundary layer, within which lie bacteria, fungal spores and arthropods. Depending on the organism, the boundary layer can be 2–3 mm thick, or 10 mm, or 300 mm. The great variability within the so-called greenhouse microclimate bears little relation to the conditions at the leaf surface such as cucumber leaves at 3 m from the ground (Jewett and Jarvis, 2001). Those boundary-layer microclimates are almost impossible to monitor directly by instruments, however tiny, without disturbing them. At best they can only be monitored remotely, or inferred by assumption and calculation. It should be possible to manipulate the microenvironment to manage a disease, by delaying the onset of dew for example, through computer-driven environment controls. However, the conditions provided for managing the disease may interfere in other aspects of the greenhouse ecosystem, e.g. other biological controls, such as parasites and hyperparasites, occupy the same ecological niche and may have different environmental requirements.

Challenges in bringing the biocontrol product to market

Even though Sporodex® has been registered in Canada and the USA as a bio-fungicide for management of powdery mildew diseases on greenhouse cucumbers and roses, there have been delays in getting the commercial product on the market for growers. This frustration points to a number of valid and practical challenges that need to be addressed by focusing on economics and industrial research in the context of biological control of plant diseases.

Development, registration and commercialization of a microbial product for disease control are expensive propositions. Cost recovery is foremost in the mind of the industrial collaborator. Therefore, the final product may be more expensive than use of standard chemical fungicides and other disease control procedures. Market capacity is a major consideration. In this regard, prior to commercialization, one must consider the value and acreage of the crop to be protected, the practicalities of delivery of the biocontrol product, its sustainability as a protective or curative control agent over the cropping season and after, and its susceptibility to negative environmental factors outside the control of the grower. For these reasons, we have targeted powdery mildews of high-value horticultural crops grown under environmentally controlled greenhouse conditions. Local, national and worldwide markets must be considered. Disease targets and plant commodities can be expanded and product availability might be enhanced by minor use registration.

Technologies and production facilities for bulk fermentation and formulation of the microbial agent can be major stumbling blocks for industrial collaborators. There are still very few companies in North America and Europe which one can contract liquid and solid-state fermentation of microbial biocontrol agents and their formulation into granular, powder and liquid biocontrol products. Serious consideration must be given to quality control (products free of microbial contamination) and satisfactory shelf-life, storage and handling by grower clients.

Environmental impacts and risk assessment are less of a concern for greenhouse users. Narrow disease specificity reduces the risk of negative non-target impacts. *In vitro* tests have indicated that Sporodex® (*P. flocculosa*) could also kill other foliar pathogens, like *Botrytis cinerea*. The commercial possibilities are now being explored. More research is required to test this product on powdery mildews and other fungal foliar pathogens outside the greenhouse and on other crop plants. We now have molecular tools and markers to identify and track the establishment, spread and survival of this biocontrol yeast and its interactions with hosts and non-target microorganisms at and beyond the site of application. Again, more ecological research on *P. flocculosa* and advances in formulation technology may allow us to expand the usefulness and market potential of this biocontrol agent for management of diseases other than powdery mildew.

Finally, we need to dispel the mindset of using biocontrol products as 'biofungicides' with a singular 'silver bullet' approach to disease control. Many biocontrol products will never satisfy the requirement imposed on chemicals to be 95–100% effective. Lesser levels of efficacy are satisfactory because many growers are now taking an integrated disease management approach involving more than one control measure. In this regard, we know that Sporodex® is compatible

with selected fungicides (Benyagoub and Bélanger, 1995; Bélanger and Benyagoub, 1997; Bélanger *et al.*, 2002).

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26

Potential and Limitations of *Microsphaeropsis ochraceae*, an Agent for Biosanitation of Apple Scab

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Overview: Apple scab, caused by the fungus *Venturia inaequalis*, is the major disease affecting apple production. The fungus destroys the marketability of fruit and can defoliate trees. To minimize the disease, growers apply almost weekly sprays of fungicides. A mycoparasite, *Microsphaeropsis ochracea*, was shown to kill the resting structures of *V. inaequalis* and thereby reduce disease in the subsequent crop by lowering the initial inoculum. This chapter describes the benefits and challenges in developing this fungus into a commercial product and marketing it as a sanitation and control measure.

Biosanitation

The high use and reliance of modern farming on pesticides is a concern both for its expense and for its environmental impacts. Practices that reduce pesticide reliance thus have multiple benefits. Sanitation is an effective method to control plant diseases caused by pathogens that overwinter in plant debris left from the summer. Sanitation includes all practices that eradicate or reduce pathogen inoculum and thus subsequent disease development. Biosanitation can be defined as the use of biological control agents to reduce the production of initial inoculum. Sanitation, including biosanitation, can be a highly effective preventative measure for many fruit and vegetable disease problems.

Research on apple scab is an ongoing activity at the Horticultural Research and Development Centre of Agriculture and Agri-food Canada. I (O.C.) was hired to replace a plant pathologist and to continue the programme on biological control of apple scab. I decided to pursue the potential benefits of 'Biosanitation' on reducing the impacts of this disease on apple production as the main theme of my work.

History of Research on Biological Control of Apple Scab

Apple scab caused by the fungus *Venturia inaequalis* (Cke.) Wint. is the major disease of concern to apple producers. Although much effort has gone into developing scab-resistant varieties, most apple cultivars grown today are susceptible to scab (MacHardy, 1996). It is virtually impossible to achieve a profit in apple production without good scab control. The development of chemical fungicides in the late 1940s launched a new era for apple production, and fungicides quickly became the primary means of scab control (MacHardy, 1996). As a consequence, little effort has gone toward developing alternative strategies. Nevertheless, for more than 50 years, scientists have viewed biological control as an alternative strategy.

Inspired by the outstanding accomplishments obtained with antibiotics, researchers in the 1950s collected microorganisms from apple leaves with the hope of finding antibiotic producers (Cinq-Mars, 1949; Simard *et al.*, 1957). Unfortunately, most of the apple leaf inhabitants produced fungistatic rather than fungicidal compounds. In the 1960s, scientists explored the use of naturally occurring saprophytes to reduce primary inoculum (Hirst and Stedman, 1962; Crosse *et al.*, 1968; Hislop and Cox, 1969). Two decades later, Andrews and collaborators (1983) made a new collection of apple leaf inhabitants and evaluated their effects on *V. inaequalis* vegetative growth and conidial germination. From these experiments, they identified an antagonist, *Chaetomium globosum*, and suggested its activity was due to nutrient competition and antibiosis. During the same period, Heye (1982) and Heye and Andrews (1983) screened fungal antagonists for their ability to inhibit pseudothecia development rather than vegetative growth and identified an effective organism, *Athelia bombacina*.

From the Laboratory to the Orchard

With the aim of developing a commercial product, Cullen and collaborators (1984) evaluated the potential of *C. globosum* for controlling apple scab. Weekly or biweekly applications reduced apple scab by only 20%, making this antagonist unmarketable. This lack of efficacy was later explained by the short life of the antibiotics produced by *C. globosum*. In contrast, *A. bombacina*, when evaluated under orchard conditions, reduced the primary inoculum of *V. inaequalis* from 60 to 100%, depending on the amount of antagonist inoculum used (Heye and Andrews, 1983; Miedtke and Kennel, 1990; Young and Andrews, 1990; Carisse *et al.*, 2000). However, the high doses of antagonist required to achieve significant scab control, combined with the high cost of large-scale production of Basidiomycetes, precluded the development of this agent as a commercial product.

To this point, the use of fungicides was perceived as the only economical control measure for apple scab control. With the introduction of new and more expensive molecules, such as the strobilurin-based fungicides, along with more rapid development of fungicide resistance in populations of *V. inaequalis*, and increasing appreciation of environmental costs and consumers' negative perceptions

of fungicides, a new focus on introducing biological-based disease control alternatives emerged (Smith *et al.*, 1991; Schneider and Dickert, 1994; Carisse and Pelletier, 1994; Bower *et al.*, 1995). Developing a biofungicide that can be used in a similar manner to chemical products is difficult. Fungicides are applied to control the fungal infection on the foliage and fruit. However, it is very difficult to attack *V. inaequalis* in its parasitic phase with a biological agent compared with when it is in its saprophytic phase. Furthermore, it will be difficult for a biofungicide to compete with chemical fungicides on the basis of cost and efficiency.

A New Programme is Launched

In 1996, a new programme was launched at the St-Jean-sur-Richelieu station of AAFC to develop a biofungicide for use as part of an IPM programme to reduce primary inoculum of *V. inaequalis*. Of the antagonists tested for their ability to inhibit pseudothecia development and consequently to reduce the production of ascospores by *V. inaequalis*, *Microsphaeropsis* sp. strain P130A was consistently the most effective. In *in vitro* tests this isolate reduced ascospore production by up to 98% and in apple orchards by 75–85%. Encouraged by these results, the company Philom Bios became interested in developing strain 130 as a commercial microbial fungicide. Agriculture and Agri-Food Canada applied for both US and Canadian patents and a 5-year research agreement was signed with Philom Bios in 1997.

The Challenges and Frustrations in Developing *Microsphaeropsis ochracea* as a Biofungicide

The development of this microbial fungicide was a mixture of challenges and frustrations. Studies on efficacy, host–parasite relations and mode of action were conducted at AAFC Research Center in St-Jean-sur-Richelieu. Studies on mass production and formulation were conducted at Philom Bios in Saskatoon. Economic analyses by Philom Bios indicated the field rate of strain P130A should not exceed 10^{11} spores per hectare. This rate became the acceptable norm for field trials, and coming to this point was an essential step in the continuation of the project. In the meantime, conclusive identification of this antagonist continued at the International Mycological Institute. Taxonomists here identified the fungus as *Microsphaeropsis arundinis* but they also concluded that because this genus was not adequately described, it may be a new species. We felt that due to major morphological differences between *M. arundinis* and isolate P130, the fungus indeed was a new species and named it *M. ochracea* (Carisse and Bernier, 2001).

Initial liquid-culture fermentation processes tested for producing spores of strain P130A resulted in extremely low spore yields, a result which is not uncommon in the development phase for many fungi. Subsequent experiments on Petri plates demonstrated that light was required for the induction of sporulation for P130A. However, it is difficult to make light available in the interior of fermentation vessels.

Therefore the production process was shifted to thin-bed solid-state fermentation. We then encountered another problem, in that this fungus produces clusters of spores in enclosed structures called pycnidia. A post-fermentation processing that releases individual spores from the pycnidia had to be worked out.

Product registration has to be an integral part of the production process development plan. For example, the harmonized regulations for the US Environmental Protection Agency (EPA) and the Canadian Pest Management Regulatory Authority (PMRA) require that we submit data from five pilot-scale production runs in the registration submission. Consequently, the time and cost of these production runs had to be factored into the development costs of the biocontrol product.

The economics of the production process had to be constantly reassessed as new information became available during development. Our initial cost estimates were conservative as we were aware that large-scale fermentation yields may be lower than lab-scale yields. Data from larger-scale runs allowed us to refine this number. The amount of spore processing required also had to be taken into account. A processing step to release spores from pycnidia may incur a 10% spore loss. If a drying step is required to produce a powdered product, spore losses may be 10–40% across the drying stage. If post-fermentation processing losses become too high, then we would have to spend more effort and money on increasing fermentation yields. The experience of Philom Bios staff with the phosphate inoculant JumpStart showed that some development can be delayed until after the product is on the market. For example, JumpStart was originally packaged as a frozen powder, but later research allowed for introduction of more user-friendly formulations. This phased approach was accepted as part of the development of *M. ochracea* strain P130A. Revenues from initial sales can support further research. Other process costs which are often overlooked in evaluating the economics of producing a biological product include the costs of packaging materials, shipping and profit margins for distributors and retailers. These were all included in determining the economics of producing the biocontrol product containing P130A.

The development of the production process must also include development of quality-assurance procedures, and these must be included in assessing the economics of the production process. Quality-assurance assays based on biological function may be complex, time consuming and labour intensive. A quality-assurance assay based only on number of viable organisms often requires 5–7 days, and the cost of delaying the release of a batch of product can be costly. This delay between manufacture and release on production scheduling and inventory management has to be accounted for when planning commercial operations.

Challenges in Bringing the Biocontrol Product to Market

The development of a biocontrol agent is a long process, usually much longer than the funding received from research grants. As a consequence, it is necessary to obtain several grants on the same project to sustain all the required research. Finding the right partners is an essential but difficult step. Ideally, restricting the development group to two partners would have been the most efficient process,

but AAFC and Philom Bios needed a third partner to help with marketing and registration. AAFC had the expertise and facilities for field testing and was able to efficiently conduct physiological studies. Philom Bios was able to produce and formulate the product and develop quality-assurance procedures. Neither AAFC nor Philom Bios, however, had the ability to market the product in eastern Canada and the USA, or the experience and resources needed to deal with the registration process. The number of companies interested in biological control products is very small. Large companies were not interested in the small sales volumes associated with this product, and small companies did not have the financial resources needed to fund all the toxicology tests that were required for registration. Once a third partner was found, it was important to have the business and legal agreements in place as quickly as possible. These agreements, especially a confidentiality agreement, enabled the partners to talk openly and establish realistic timelines and research goals. Face to face meetings were a crucial step in securing the agreements and planning the project.

The registration process remains one of the major challenges in bringing this product to market. The product will be sold in both Canada and the USA, so both jurisdictions must be considered. Canada's PMRA and the USA's EPA have a joint registration process. Early consultations with both the PMRA and EPA defined what tests would be needed. This required, and continues to require, clear communication and negotiation between all parties. At times, the requirements that were originally developed for chemical pesticides do not seem to make sense for biological products. The registration process does slow the development of the product, and the time needed to obtain registration had to be factored into the calculations on the economic payback for the product.

Implementation and Growers' Acceptance

Right from the beginning of the research, we decided that the biological control strategy we developed needed to be integrated into a disease management programme. It was clear that the objective was not to replace chemical fungicides but to complement conventional disease management programmes. When the concept of the product was presented to apple growers, most agreed with the idea that reducing pesticide usage was to their benefit. However, the tolerance threshold for apple scab is around 1% scabby fruits. Above this threshold, profits are reduced due to picking cost increases and reduced quality and storability. As a consequence, control of apple scab (biological or not) must be extremely effective and must be able to compete with all available fungicides.

In practice, a biological product is unlikely to have all the characteristics of modern fungicides, such as the ability for application under diverse climatic conditions, high leaf adherence and rapid eradication capability. Unless apple growers can receive a market premium for apples produced with 'less pesticides' or using 'IPM practices' they will not replace chemical fungicides by a biological one. Despite these hurdles the concepts of 'biosanitation' remain attractive to growers for several reasons. By integrating a biofungicide that reduces initial inoculum into their production systems, they reduce the risk of scab control failure,

which occurs under high disease pressure. Furthermore they increase the potential to significantly reduce the number of fungicide applications. Because the product is sprayed in the autumn (or late summer), growers can still rely on their conventional programme the following spring to manage the disease. It is likely that at first growers retain the same fungicide programme the spring following an application of a biofungicide but as they become more accustomed to the impacts of the product, they will probably eliminate unnecessary chemical treatments. Apple growers have called this approach their 'insurance policy'. They may not need to use it every year, but in years when scab pressure becomes very high, the biofungicide will allow them to bring the inoculum to a manageable level. Technically, they see several advantages to biosanitation. In the autumn fungicide residues on crops are generally not an issue. The window for a single application is broad enough to allow for integration with other practices and selection of the most ideal weather conditions (Carisse and Rolland, 2004).

Biosanitation is easily combined with other sanitation practices to reduce inoculum, especially in orchards that were not well managed for scab the previous year. Sanitation practices, including flail-mowing fallen leaves in autumn and application of urea to fallen leaves, can reduce apple scab inoculum by 50–75% (Sutton *et al.*, 2000) and to up to 80–90% when combined with application of *M. ochracea* (Vincent *et al.*, 2004). Sanitation practices including biosanitation will not be cost-effective unless they reduce scab management costs or yield losses during the growing season. To attain a significant reduction in cost of fungicide, sanitation and biosanitation practices are generally combined with a delayed fungicide programme (Carisse and Rolland, 2004).

Some conclusions

Although *M. ochracea* was tested under orchard conditions and proven useful in apple scab management, it is not yet on the market. When it will be available to apple growers is hard to say. All ingredients for success are there: *M. ochraceae* provides consistent efficacy of 80–90%; the expected cost will be affordable (compared to available fungicides); apple is a high-value perennial crop; and apple scab is a major disease of apple. There is also good potential for use of this biocontrol product on other systems, such as *Rhizoctonia solani* on potato tubers and Botrytis leaf blight caused by *Botrytis squamosa*. Nevertheless, the product will be commercialized only if there is a substantial amount of time, money and commitment invested.

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27 Competitive Exclusion of Aflatoxin Producers: Farmer-driven Research and Development

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Overview: Aflatoxins are highly toxic, cancer-causing chemicals produced by fungi belonging to the genus *Aspergillus*. *Aspergillus flavus* is the most important causal agent of crop aflatoxin contamination. We developed a strategy for preventing aflatoxin contamination based on the use of naturally occurring isolates of *A. flavus* that lack aflatoxin-producing ability (atoxigenic strains). The atoxigenic strains displace aflatoxin producers during crop development and infection and thereby reduce contamination. Although significant single-season effects are achieved, the greatest potential is for long-term and area-wide influences. This chapter discusses the history of development and commercialization of atoxigenic strains.

Aflatoxin Contamination

Aflatoxins are highly toxic, cancer-causing chemicals produced by several fungal species within *Aspergillus* section Flavi. Presence of aflatoxins in human foods causes acute and chronic health effects (aflatoxicoses), ranging from immune-system suppression, growth retardation and cancer to death from acute poisoning (Wild and Turner, 2002). In developed countries, stringent government regulations limit the use of aflatoxin-contaminated crops in foods and feeds, and, as a result, commodities with aflatoxin content exceeding the maximum permissible level have significantly diminished cash value. In crops intended for human consumption, maximum permitted aflatoxin levels range from 2 ppb in the European Union to 20 ppb in the USA. As aflatoxins are readily transferred from animal feed to milk, similar stringent regulations are imposed on feed intended for dairies (Wu, 2004). The action level for aflatoxins in US milk is 0.5 ppb.

The presence of aflatoxins impacts the US cotton industry because dairies pay a premium price for cottonseed, and the cottonseed must contain less than 20 ppb to enter that market. In areas with severe contamination, it is not unusual to find cottonseed containing in excess of 1000 ppb of aflatoxins. Crops having over 300 ppb are prohibited from being fed to any animal in the USA. Furthermore, the use of rotation crops susceptible to aflatoxin contamination, i.e. maize and groundnuts, is limited by the frequency and severity of aflatoxin contamination in the warm cotton-producing regions of Arizona and South Texas (Cotty, 2001). Aflatoxin contamination frustrates farmers, middlemen and processors alike. The crop may have no visible symptoms and yet contain sufficient levels of aflatoxins to either reduce its value or completely exclude it from the market. The same fungi can cause contamination of several crops grown in a given area, including cottonseed, maize, groundnuts and pecans in south Texas. Although, the conditions favouring contamination may not be the same for all crops, culturing any of these susceptible crops, as well as non-susceptible crops, influences the communities of aflatoxin-producing fungi resident in fields (Orum *et al.*, 1997) and thus the fungi the next crop will be exposed to.

Development of Atoxigenic Strains as Biocontrol Agents: the Initial Concept of Biological Control

Biological control was not the initial target of the research; the goal was to obtain a greater understanding of the contamination process and to develop improved variety recommendations and agronomic practices in order to reduce the vulnerability of the crops without costly inputs. However, even when optimal cultural practices and ideal cultivars are utilized, farmers still experience unacceptable aflatoxin contamination when environmental conditions favour infection by *Aspergillus flavus* (Cotty, 2001), the primary cause of aflatoxin contamination on cottonseed and maize. Temperature ranges of 30–38 °C and high humidity conditions are ideal for multiplication of *A. flavus* on organic material (i.e. plant, insect and mammal debris). The fungus exists as complex communities that can be divided into distinct morphotypes (commonly called strains) and numerous vegetative compatibility groups (VCGs) (Cotty, 1989; Bayman and Cotty, 1991). The strains and VCGs vary widely in aflatoxin-producing ability (Cotty, 1989; Bayman and Cotty, 1993), and thus the aflatoxin-producing potential of fungal communities differs among regions and even fields (Cotty, 1997). A distinct morphotype of *A. flavus*, called the S strain, was found to have a major role in the contamination of cottonseed with aflatoxins (Cotty, 1989). Interestingly, the capacity of the S strain for aflatoxin production was not related to its virulence as reflected by ability to rot bolls and ramify through tissues. This suggested that isolates lacking aflatoxin-producing ability (atoxigenic strains) might be able to compete with, and possibly exclude, aflatoxin producers. In doing so, atoxigenic strains would reduce infection by aflatoxin producers and thereby aflatoxin contamination (Cotty, 1989).

Greenhouse studies

Greenhouse studies confirmed that certain atoxigenic strains of *A. flavus* reduced infection and aflatoxin formation by aflatoxin producers when the two strains were co-inoculated on to plants. Some atoxigenic strains were more effective than others (Cotty, 1990) and some could not inhibit aflatoxin formation (Cotty and Bhatnagar, 1994). The most effective isolate, *A. flavus* AF36, consistently reduced contamination by over 90% when applied at inoculum concentrations equal to the aflatoxin producers (Cotty, 1990). When AF36 was applied either at one tenth the conidial concentration of the aflatoxin producers or one day after, there was still a significant reduction in toxin levels (Cotty, 1990). The primary mode of action was shown to be exclusion by competition during ramification of host tissues and competition for nutrients (Cotty and Bayman, 1993). The influences on aflatoxin content occurred without increases in either boll decay or spread of the fungus through the host tissues (Garber and Cotty, 1997). Individual seeds and bolls of cotton are typically infected by multiple VCGs and/or strains (Bayman and Cotty, 1991) and AF36 turned out to be a common VCG isolated from infected cottonseed. Thus, the effect we observed with the atoxigenic strain under greenhouse tests is a natural phenomenon. The use of natural atoxigenic strains to reduce contamination seeks to increase the frequency of this natural phenomenon. Even before initial greenhouse experiments were written for publication, industry groups (the National Cotton Council (NCC), National Peanut Council, and National Cottonseed Products Association) were pushing for field tests of the concept of utilizing atoxigenic strains for reducing contamination.

Field studies

In addition to interfering with aflatoxin contamination during coinfection of crops, atoxigenic strain applications seek to displace aflatoxin producers from the crop environment. The composition of *A. flavus* communities in individual fields, however, shifts during and between seasons, independent of any external intervention (Bayman and Cotty, 1991). This indicates that founder effects are common and that the original colonizers are key players in determining the structures of *A. flavus* communities on annual crops. *A. flavus* populations decline under unfavourable climatic conditions but build rapidly when favourable conditions return. Such cycles coincide with both seasons and crops. Applications of atoxigenic strains were timed to coincide with the beginning of conditions that favour establishment of *A. flavus*, thereby facilitating the establishment of the applied strains. It was hoped that with this approach atoxigenic strains would become dominant members of the fungal community on developing crops.

As commercial field studies expanded, it soon became apparent that although direct interference with aflatoxin production by an atoxigenic strain during crop infection may be important and one aspect of field efficacy, other aspects were at least equally important. Properly timed applications of relatively small quantities of atoxigenic strains were found to shift the composition

of *A. flavus* communities without increasing either the quantity of fungus on the crop or the amount of the crop infected. Thus, a single application of 10 lbs per acre of colonized wheat seed (Fig. 27.1) shifted the proportion of atoxigenics from 1–2% of the total *A. flavus* community on the crop or in soil to around 80% of the community (Cotty, 1994; Cotty and Antilla, 2003). Displacement of aflatoxin producers throughout the environment turned out to be both an important aspect of atoxigenic strain activity and a basis for efforts to develop area-wide strategies to reduce vulnerabilities of all crops produced in treatment areas to aflatoxin contamination. Large and significant changes to the incidences of atoxigenic strains in the environment and the resulting reductions to the average aflatoxin-producing potential of *A. flavus* communities are achieved without increasing the overall quantity of *A. flavus* on the crop at harvest, in the soil, or in the air (Cotty, 1994; Cotty *et al.*, 1994; Bhatnagar *et al.*, 2001; Cleveland *et al.*, 2003; Bock *et al.*, 2004).

Positive influences were, in fact, frequently achieved in fields adjacent to and/or nearby treated fields. However, just as the atoxigenics move to neighbouring fields, so do the aflatoxin-producing strains, thereby eroding the long-term benefits. Thus, we consider the greatest potential for atoxigenic strain use to be area-wide management programmes. For such a control strategy to be effective the biological control agent must have ecological competence and be able to increase to epidemic proportions when its activity is most needed.

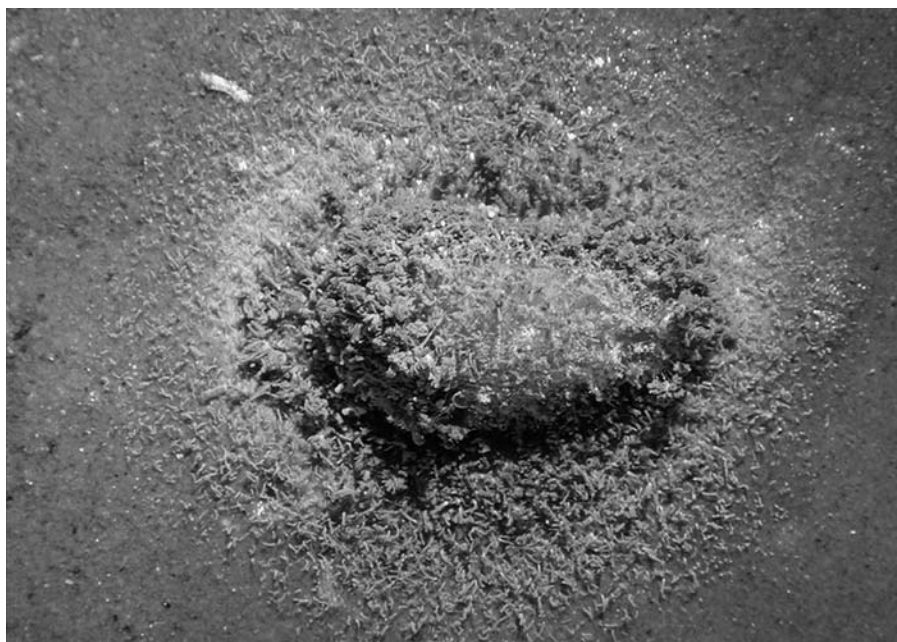


Fig. 27.1. Atoxigenic strain *Aspergillus flavus* AF36 growing out from product (colonized wheat seed) on soil 7 days after application.

Government/Industry Partnerships

In 1988 severe aflatoxin contamination of crops within the US Corn Belt came to the attention of the press and the US congress (Cole and Cotty, 1990; Robens *et al.*, 1990). Representatives for several US crops used this opportunity to leverage additional research funding for developing novel methods to prevent future outbreaks. The initiative was centered on annual workshops where progress was evaluated by both researchers and industry members of the Multi-crop Aflatoxin Working Group (MCWG), which was composed of representatives of crops affected by aflatoxin contamination including cotton, maize, groundnuts and tree nuts (Robens *et al.*, 1990).

At the initial Aflatoxin Elimination Workshops, researchers introduced the idea of using natural isolates of *A. flavus* that did not produce aflatoxins (atoxigenic strains) to competitively exclude aflatoxin producers, along with strategies for utilizing recently developed technologies for improvement of host resistance (Cole and Cotty, 1990). Interest and involvement of agro-industry would turn out to be central to applying biocontrol based on atoxigenic strains of *A. flavus* to commercial fields. Information on competition during groundnut infection between aflatoxin producers and an *Aspergillus parasiticus* isolate that produced a toxic aflatoxin precursor but not aflatoxins was also included at the first workshop (Cole and Cotty, 1990). The same groundnut researchers later adopted use of natural atoxigenic *A. flavus* strains and developed one into the product marketed as Aflaguard (Dorner, 2004).

At the outset the concept of using atoxigenic strains to limit aflatoxin contamination was highly controversial and not well received by researchers. Contentious exchanges surrounding the concept were even documented in the Wall Street Journal (Kilman, 1993). The project may have stopped there as an interesting paper if it were not for the industry's interest in advancing aflatoxin management. Although breeding for resistance and transgenic crops continued to receive much greater attention at all workshops as likely control methods, representatives of both the groundnut and cotton industries maintained intense interest in developing atoxigenic strain technology for over 15 years (Robens and Riley, 2002).

Attempts to license atoxigenic strain technology for commercial development were hampered by lack of information as to the value of such products to the target users. There was no example of a product that prevented pre-harvest aflatoxin contamination, and there was concern over the liability associated with product failure. Thus, when in 1998 the Arizona cotton industry decided to pursue development of atoxigenic strain technology, ARS viewed it as the most viable alternative for continued development and expansion of aflatoxin control strategies based on atoxigenic strains. The partnership between ARS and the cotton industry succeeded in bringing this technology into commercial reality and continues to improve the product for use under diverse conditions. In excess of 50,000 ha of cotton have been treated with atoxigenic strains in Arizona, Texas and California. While interest in employing this strategy exists for many other crops, it is the economics of contamination that will ultimately decide the extent to which this technology will be used.

Industry partnerships

The extent to which atoxigenic strain technology came to be developed is a direct reflection of US cotton industry involvement. This includes commitment of individual farmers and gins, long-term and continuing commitments of the Arizona Cotton Growers Association and NCC, direct funding from the Cotton Foundation, Arizona and Texas State Support Programs of Cotton Incorporated, the Texas Cottonseed Crushers Association, the National Cottonseed Products Association, and assistance in obtaining other sources of funding. Farmers, gins, crop organizations (i.e. South Texas Cotton and Grain Association) and oil mills (i.e. the Valley Coop Oil Mill in Harlingen) all participated and funded operations on their farms, providing labour, application costs, time, personnel and resources. Farmers frequently helped with crop, soil and air sampling. Participation by farms was voluntary and changed with shifts in economics and needs. Close relationships were forged by frequent interaction with diverse industry members. Research always included fieldwork and trips to farms, gins and oil mills, which led to many fruitful discussions. We participated in diverse industry meetings and provided advice and assistance on mycotoxin problems. The limitations of biocontrol and the economic constraints under which it functions were frequently included in discussions. The diverse relationships led to a strong partnership between farmers, farmer-run organizations, the University of Arizona and the Agricultural Research Service, which became the driver for the direction of the research programme.

The cotton industry representatives quickly realized that this strategy was a potentially practical, realistic approach to a long-standing and seemingly unsolvable problem. This was particularly true in Arizona, where high levels of aflatoxin in cottonseed reduced grower profitability year in and year out. Multi-year field tests of atoxigenic strain technology in Arizona showed great promise in reducing aflatoxins (Cotty, 2000; Cotty and Antilla, 2003). As a result, in 1998, the board of directors (all farmers) of the Arizona Cotton Research and Protection Council (ACRPC), an organization funded by check off-funds, voted to add development of atoxigenic strain technology as part of their responsibilities. Discussions resulted in an unusual partnership, where Cotty's ARS laboratory would develop data required to obtain a pesticide registration for strain AF36, develop protocols and a facility for manufacturing the atoxigenic strain material, and develop procedures of practical value in running an aflatoxin management programme. The NCC would assist ARS and ACRPC in working with the IR-4 project and EPA to get registration of AF36. Because ACRPC had extensive experience with area-wide pest management, it would assist with research activities. ACRPC was indispensable as a resource in the development of atoxigenic strain technology at the commercial scale.

Assisting farmers with bringing a new biocontrol agent into use is a huge endeavour. Farmers are busy people and it is essential to maintain their enthusiasm during long periods over which biopesticide development may occur. Site visits by our researchers to farms and gins were essential for maintaining industry interest. Even today, participants want detailed reports on how the product is performing and how their results relate to those of others. As the number of

participants increased it became increasingly difficult to see everyone as often as desired. In retrospect, setting up more local contacts who could be involved from the start would have allowed better feedback to farmers, as their involvement was the most crucial in the proof of concept and uptake of the technology over a large area. Those farmers that paid close attention to details such as the timing of applications and subsequent field operations got remarkable results. One such farmer applied the atoxigenic strain at half the recommended rate in order to double his treatment area and as a result of his close attention to detail, he obtained excellent results.

Perseverance, an essential ingredient

Public sector partnerships require patience, endurance, multiple contacts and a willingness to compromise. Over the decade that it took to commercialize our product several key supporters and collaborators died, went out of business, or stopped supporting our work. Perseverance meant carrying out experiments that provided answers to questions regardless of any extraneous issues. Experiments were designed to give results regardless of agricultural activities and regardless of industry adherence to agreed-upon protocols. Our goal was to obtain results each year and not to emphasize sub-optimal aspects of collaborations or blame collaborators for not meeting publication needs. This helped to build grower–researcher teams. We first focused on developing protocols that allowed farmers to use our technology in a manner that fitted their farming practices. Only then did we ask them to alter their practices in a manner that would lead to maximum disease control efficacy. There is much to learn inside the farm gate; not all fields and regions are the same and biologicals do not perform the same in all locations.

Commercialization, formulation strategy and manufacturing

The experience of producers with transgenic technology in cotton greatly influenced the approach to developing our biocontrol technology. Farmers felt purveyors of *Bt* and herbicide-resistant transgenics extracted maximum profit and limited producers' ability to gain financially from the technology. Some were reluctant to adopt transgenics because of this perception. As a result, all farmers lost out on environmental and area-wide pest management benefits. It was hoped that the biocontrol technology could be held in the public sector with most of the economic benefits kept within the farm gate. Toll manufacturers were seen as entities that could potentially influence profitability and access, two aspects the farmers did not want to relinquish. The growers wanted assurances that they would be able to retain control of both costs and manner of implementation. By developing a facility governed by a farmer-run organization, we hoped to meet both these concerns. The production technology appeared simple at the lab scale. Autoclaved wheat was inoculated with a spore suspension of an atoxigenic isolate and after a short incubation it was dried (Bock and Cotty, 1999). This had the

theoretical advantages of being a pure culture and also providing the fungus with an available food source (Bock and Cotty, 1999). There are diverse atoxigenic strains, and such a product allows for rapid and easy mixing of batches and the potential to inexpensively customize formulations for specific locations or crop rotations.

Naively, we relied on engineering consultants from the pharmaceutical industry and equipment sales staff as our resources for development of the commercial facility. After a year we abandoned the systems specialists and pursued development empirically and intuitively by gradually improving each process step. Sometimes equipment not available off the shelf was designed and custom manufactured. Most of these pieces, or derivatives thereof, are still in use today. The lack of any public sector facilities from which to borrow expertise was a limitation to our efforts. Fortunately Cotton Incorporated provided engineering expertise in grain-handling systems, and APHIS donated the services of Joe Ploski, with experience in constructing and running sterile insect facilities, to help with facility design and in troubleshooting.

We used wheat in our formulations because it was relatively inexpensive and readily available. Other grains, however, would have worked equally well and some offer advantages under certain circumstances (Bock and Cotty, 1999). The philosophy was to make the product inexpensive and axenic. We also wanted the product to be locally produced and under farmer control. Researchers at the National Peanut Research Center took a different approach in developing what became the product Aflaguard marketed by the private company Circle-One Global. This product uses unsterilized rolled barley with sufficient quantities of spores to overcome any microbes on the seed. The spores were custom produced by a toll manufacturer (Dorner, 2004).

The ACRPC-ARS facility that produces the atoxigenic strains (Fig. 27.2) uses a simple process that could be adopted for production of multiple microorganisms of regional importance. This would allow for facility expenses to be bridged across several products. Simplicity was the design goal for both the facility and the formulation. A stable product was sought that would allow manufacture months before use and provide some grace period during which it could remain in the field after application awaiting conducive conditions (i.e. irrigation or rain). The developed commercial-scale process uses minimal personnel, and currently only two people carry out all the steps from spore production, quality control, manufacturing and packaging to shipping. The atoxigenic strain products are easy to use in the field and easy to transport.

Assessment

When aflatoxin contamination is low it can be attributed to high product efficacy, whether or not the biocontrol agent had any effect. The opposite is true when disease levels are high. Aflatoxin contamination is highly variable. The variability is such that stringent sampling and analytical procedures are required to measure the crop toxin content. This causes aflatoxin contamination to appear mysterious and growers instinctively view aflatoxin numbers with scepticism. We set out to provide growers with an independent measure of the extent to



Fig. 27.2. The manufacturing room of the atoxigenic strain manufacturing facility developed by a partnership between the Arizona Cotton Research and Protection Council (ACRPC) and the USDA Agricultural Research Service.

which the control agent influenced aflatoxin concentrations. Since the first commercial field treatments in 1996, extensive microbiological analyses have been performed to meet this goal. Currently, well over 10,000 *A. flavus* isolates per year are collected from soils and crops and characterized by vegetative compatibility analyses (Cotty, 1994). This provides an assessment of the extent to which treatments modified the community structure of *A. flavus* resident in each area receiving treatments. The analyses are routinely performed by both ARS and in an industry (ACRPC) laboratory built and run for the purpose. Growers, gins and other participants are provided with data allowing them to follow effects of the control agent on the fungal community and to assess if the management programme is progressing in an acceptable manner. The analyses provide the actual proportion of *A. flavus* community represented by AF36. These analyses allow influences of applications to be evaluated in both low toxin years and in years when most toxin levels exceed the regulated 20 ppb level. Such analyses and the resulting reports to collaborators greatly helped the survival of this biocontrol strategy.

Economics

Laws regulating the limits of contamination in crops are such that a highly effective application may provide no economic benefit under one circumstance whereas

poor control may provide a significant gain under another circumstance. Furthermore, the economic benefits may spread over many years due to residual effects, and treatments frequently have beneficial influences beyond treated fields. Such effects make economic impacts a moving target. The greatest impact on contamination can be obtained when the product is applied yearly to a large area. This reduces the aflatoxin-producing potential of fungi resident throughout the entire agroecosystem. A region (gin, oil mill, grower organization) entering into an area-wide programme having 80% of its crop above 20 ppb may expect a three- to fourfold return on its investment. However, after several years of treatments, incidence of unacceptable contamination will have dropped. Once incidence of unacceptable contamination dropped to below 20%, return on investment would drop to less than the actual cost of treatment. Farmers then may decide to forgo treatments until aflatoxin levels return to high levels. Unfortunately, aflatoxin values are so variable between fields and across years that one can never predict in which direction toxin levels will go. Economics were not a concern during the research phases of this programme but as the product matured decisions to continue to treat became an issue. Farmers do not forgo herbicides once weeds are brought under control, because the weeds would soon re-establish. Why the same logic is not applied to beneficial microbes is a good question.

Biopesticide Registration

While efficacy issues around releasing atoxigenic strains were of concern, they were minor compared to the perceived notion that this strategy posed an outright environmental threat to crops and people. The strains to be released were intended to be highly competitive and to retain virulence to crops. In addition, *A. flavus* was known to be allergenic and to infect immune-compromised patients. In 1993, early in the development of concepts and 3 years before receiving the first experimental use permit (EUP), we met with the US EPA to discuss the science behind atoxigenic strain technology. Details involving registration based on the EPA perspective are provided by Bacchus (Chapter 28, this volume). The biopesticide group at EPA brought together scientists and department heads involved with biopesticide registration and listened to a detailed presentation on the biocontrol strategy and the science behind it, including information on the ecology, biology and distribution of the biocontrol agent. It was clear from this moment that although the regulatory process was somewhat rigid and time consuming, EPA was open to new types of data useful for risk assessment of biological control agents. EPA was particularly open to information on the field biology, ecology and population dynamics of the microbial system in question. This dictated, at a very early stage, that one of our goals had to be the assessment of the short- and long-term influences of applications, and, even today, this aspect influences the manner in which the product is used. In the course of obtaining information for EPA, we showed that the atoxigenic strains displace the majority of aflatoxin-producing fungi without increasing the quantity of fungus either in the environment or on the crop, and without increasing the proportion of the crop infected. Similarly, 1 year after application the quantities of *A. flavus*

propagules in the soil are not increased but the incidence of aflatoxin producers is greatly reduced. We also provided EPA with other useful data on the incidence and magnitude of aflatoxin contamination and *A. flavus* ecology in both agriculture and natural habitats. Three years after the initial meeting with EPA, an Experimental Use Permit (EUP) allowing treatment of commercial crops was issued in 1996. The EUP was expanded several times until it ultimately allowed 8000 ha of tests in Arizona and Texas. In 2003, a section 3 registration was granted for Arizona and Texas, and in 2004 this was expanded to southern California. These are the only three states with significant aflatoxin contamination of cottonseed.

The EPA registration process was reasonable, complex, and it altered the course of our technology development. It was indispensable to have IR-4 Biopesticide Managers to interpret what was needed, serve as a liaison with EPA, and help prepare and submit files. Such a programme is an incredible resource; it even provided some of the research funding needed to obtain data for registration. The research carried out for registration helped us to discover the carry-over influences of atoxigenic strains. Once influences that carried over multiple years were discovered (Fig. 27.3), an emphasis on long-term and area-wide management began. Applications of multiple atoxigenics, either simultaneously or in succession, is a strategy that might provide optimal efficacy because application of multiple atoxigenics should result in a more complex and potentially more stable fungal community structure with a greater resistance to re-establishment of a fungal community with a high aflatoxin-producing potential. Furthermore, application of multiple VCGs should create greater competitiveness in diverse micro-niches, as well as the opportunity to customize formulations for different areas. Such strategies are particularly impacted by

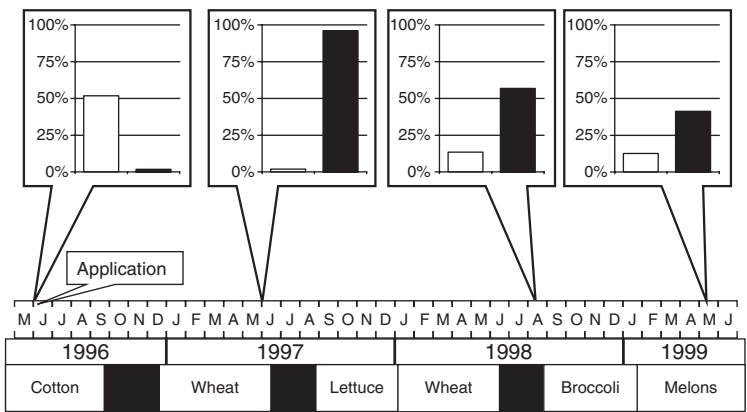


Fig. 27.3. Long-term influence of atoxigenic strain AF-36 application on the percent of the overall *A. flavus* community (y axes) resident in the soil composed of either the high-aflatoxin producing S strain (empty bars) or AF-36 itself (solid bars). Bottom bar indicates crops planted during various periods, solid portions indicate fallow. A single application of wheat seed colonized by the atoxigenic strain was made to this 40-acre field on June 4, 1996 at the label rate of 10 lb per acre. From *Managing Aflatoxins in Arizona* (Cotty and Antilla, 2003).

pesticide regulations because approval is required for each additional strain, which could eventually number in the hundreds. Development of regulations and policies that allow simple and rapid adaptation of many similar, but not identical, isolates will facilitate development and optimization of biocontrol technologies targeted to multiple crops, locations and environments.

Patents, Licences and Development

The process of patents and public versus private sector development is complex and not familiar territory for most scientists. Private company product development often can initially progress faster because of greater amounts of capital available for development and advertising. For the product considered here public sector development proved to be a useful alternative and resulted in a product available inexpensively to groups with apparently small market potentials. Although the patents protecting the atoxigenic strain served well for its development they also proved to be a hindrance. Institutional policies frequently seek revenue-generating licences with corporations that require exclusive rights. However, other avenues may provide wider application of the technology while allowing a venue for public sector researchers to build upon prior accomplishments. Efforts to expand and improve public sector use of technology, as in the current case, may more extensively benefit agriculture both in the USA and internationally. Patents cost money and public organizations feel the need to recover these costs. Recovery of such costs, not the benefits of the technology, can become the driver of licensing. In theory, good patent coverage with exclusive licence is an incentive to corporate investment. However, this strategy does not always best serve the future of biocontrol. By keeping microbial strains in the public sector, in a manner similar to plant breeding lines, we may better facilitate development of certain biocontrol technologies.

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28 Aflatoxin Control in Cotton and Groundnuts: Regulatory Aspects

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Overview: Aflatoxin is one of the most potent mammalian carcinogens known. The dietary source of this mycotoxin is food or feed colonized by strains of *Aspergillus flavus*. Research described in a related chapter showed that problematic strains of *A. flavus* could be displaced from commercial crops by non-aflatoxin-producing strains of the fungus (see Cotty *et al.*, Chapter 27 this volume). The US Environmental Protection Agency (EPA) has conditionally registered two of these non-aflatoxin-producing strains as biopesticides for cotton and groundnuts. This chapter details how information gathered from field studies on the ecology of the control agents, as well as data provided in support of the Agency's requirements, were used to register these microbial pesticides. The chapter offers insights as to the approaches used by EPA for risk management of biologically based pest control agents.

Background

Toxigenic strains of *Aspergillus flavus* produce aflatoxin, a potent human hepatic carcinogen associated with liver cancer. Losses due to aflatoxin contamination cost the US agricultural industry millions of dollars annually in rejected or lower-quality food commodities. In the arid west and south-western USA, infection of cotton by aflatoxin-producing *A. flavus* is a major problem, especially during drought conditions, when the fungus proliferates. Few alternatives, if any, exist for managing infestation of cotton or other crops by aflatoxin-producing *A. flavus* strains. Infected food material can be decontaminated via ammoniation but this is expensive and not universally available. Also, the procedure decreases the value of the treated commodity. Aflatoxin contamination in cotton can be reduced somewhat by manipulation of harvest date, costly irrigation practices, different harvest methods and storage practices. Aflatoxin contamination is generally more common under dry conditions and is a perennial disease of cotton in the desert regions of the USA. However, food commodities grown in arid tropical regions around the world are also heavily affected by aflatoxin contamination.

The details of the disease and the research are described by Cotty *et al.* (Chapter 27 this volume). Two non-aflatoxin-producing strains of *A. flavus*, AF36 and NRRL 21882, were identified as biological control agents that significantly reduced aflatoxin contamination of cotton and groundnuts, respectively. The strains AF36 and NRRL 21882 are non-aflatoxin-producing or atoxigenic strains of *A. flavus*. The genus *Aspergillus* occurs worldwide and some species produce mycotoxins. A few members of the genus have been domesticated for commercial use. For example, the product 'Beano' is made using alpha-galactosidase obtained from *Aspergillus niger*, and soy sauce and miso are products derived from the fermentation of soybean using strains of *Aspergillus oryzae*.

The Agency has classified *A. flavus* strains AF36 and NRRL 21882 as active ingredients for use in microbial pesticides. AF36, the non-aflatoxin-producing strain of the *A. flavus* fungus, is a naturally occurring strain that was isolated in Arizona from cottonseed, but is also indigenous to Texas. Both AF36 and NRRL 21882 were discovered in the same complex of fungi. They lack aflatoxin production and have a unique vegetative compatibility group which does not allow any exchange of genetic material with the aflatoxin-producing strains.

Apparently, these two strains are part of a complex of ecotypes that lack some critical enzyme required for toxin biosynthesis (Ehrlich *et al.*, 2002). The mode of action of these non-producing strains is presumed to be competitive displacement of the aflatoxin-producing strains from the crops (Cotty, 1992; Cole and Dorner, 2001). Applying even very low inoculum concentrations of the atoxigenic strains (active ingredients), in the form of a granular formulation, allows these strains to colonize the treated commodity and thereby preclude infestation by the toxic strains. Timing of application is critical in order to precede infestation by aflatoxin-producing strains. Both pesticides are applied by ground or air. AF36 is applied to cotton at the pre-bloom stage, and NRRL 21882 at the pre-pegging stage of groundnut growth.

From Laboratory to Market

Researchers proposing to register an active ingredient as a pesticide need to be fully aware of the length of time that it takes to bring a product from laboratory to the market place. With this in mind they must have in place strong alliances with growers and the commercializing partner, and commitments from them that they will stay the course. For this the scientists must convince the growers that the proposed active ingredient will be worth the effort spent on field research. Similarly, the manufacturing sector needs to be convinced to commit financial resources over the long term with the expectation that they will be rewarded when the product does reach the market. It is also imperative that issues that may affect acceptance of the product by consumers and the public be taken into account early in the process. Alliances, support and acceptability are not factors within the realm of the Agency's review of registration but without such support, and participation from the stakeholders, the chances of successful pesticide registration and implementation into agricultural practice are diminished. The efforts that

went into commercialization of strains AF36 and NRRL 21882 provide an excellent example of how stakeholder participation propelled the process of registration. At the time registration was proposed no other materials were available to control aflatoxin contamination of crops. There still is no other biopesticide registered for this use.

Breaking down barriers

The scientists who discovered the biocontrol strains had obtained patents and generated many publications on the efficacy of the organisms. However, in order to register the pesticide and use it commercially, they were required to provide data that were appropriate to meet US EPA's microbial data requirements for human and ecological safety assessment. Such ecological data are rarely collected during investigations of plant disease control. Two different approaches were taken to register these pesticides. AF36 took a microbial/ecological approach, where soil and air monitoring data were collected from field and laboratory studies. NRRL 21882, on the other hand, was conditionally registered on the basis of laboratory data and small-scale field trials. Regardless of the approach, it took many years from the inception of the idea to conditional registration of the pesticides. The key issues that needed to be addressed before registration could be granted focused on the following aspects.

1. The concept that applying strains of *A. flavus* to a crop, even though they did not produce toxins, as a means to diminish the activity of the toxigenic strains was an unusual and untested strategy for disease control. Growers, the public and scientists needed time to understand, evaluate and support the concept that benign *A. flavus* strains could control toxigenic strains. Data collected from demonstration plots in growers' fields convinced them that adding the benign strains of *A. flavus* AF36 did not increase the risk of aflatoxin contamination.
2. There was a potential that adding *A. flavus* AF36 to a field would upset the fungal ecology and potentially create other problems. Detailed examination of the microbial ecology after application of the control agents not only demonstrated its efficacy as a disease management tool but also showed that adding *A. flavus* AF36 did not change the total fungal population numbers. Such tests, however, necessitated that multiple years of field trials be conducted under Experimental Use Permits.
3. The registrants had to produce data showing that the atoxigenic *A. flavus* AF36 strains were not pathogenic to humans, birds and honeybees. Field studies examining total *A. flavus* populations in untreated and treated fields established the baseline for the naturally occurring background levels of the fungus, and the rapid drop in *A. flavus* levels to background after treatment. This aided the risk assessment process.
4. It was discovered that displacement of the toxigenic strains occurred only when contiguous cotton fields were treated. As a result, large amounts of inoculum of AF36 were required, bordering near commercial-scale production.

However, this occurred at the experimental phases of the work, when such production capacities were not readily available. The experiences with AF36 group, however, did provide insight to the scientists for what requirements were necessary for registration of the NRRL 21882 group. For the Agency, experiences with AF36 allowed for accelerated registration of this latter pesticide.

5. The extended process for registration meant that the researchers needed to maintain and continue to add to the alliances with the grower community and entrepreneurs to commercialize the products. In addition, they had to convince the US EPA and the public that registration and use of the pesticides would not cause any unreasonable adverse effects to human health and the environment.

6. Imparting information about other *Aspergillus* strains, such as *A. oryzae* and *A. niger*, which are used to produce consumer goods, helped to clarify the beneficial roles of the non-aflatoxin-producing members of the genus.

Microbial Data Requirements and Guidelines

In the USA, pesticides are registered under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Food tolerances or exemptions from food tolerances (sometimes called maximum residues limits) are granted under the Federal Food, Drug and Cosmetic Act (FFDCA) as amended by the Food Quality Protection Act (FQPA) of 1996. The data requirements for microbial pesticide registration are found in 40 CFR 158.740. Guidance to registrants on how to provide those data can be found in the Microbial Pesticide Guidelines 885 series (see Biopesticides and Pollution and Prevention Division's (BPPD's) website: http://www.epa.gov/oppbppd1/biopesticides/regtools/guidelines/microbial_gdlns.htm).

Generally, most microbial pesticides do not require more than Tier I data to support a registration. Tier I health effects data comprise: (i) toxicity/pathogenicity studies for acute oral, pulmonary instillation, intravenous or intraperitoneal injection and dermal effects; (ii) studies concerning primary dermal and eye irritation; and (iii) hypersensitivity studies and reports. The pesticide must show a pattern of clearance in exposed organs and tissues during the relevant infectivity/pathogenicity studies and must not disrupt endocrine functions.

Tier 1 data are also required for environmental effects to demonstrate whether potential unreasonable adverse effects are expected to pose a risk to birds, fish, wildlife and beneficial and other non-target insects. Aflatoxin-producing fungi, because of the toxin's potent hepatocarcinogenicity, are considered public health pests, and the efficacy of pesticides with aflatoxin control claims needs to be verified. Consequently, efficacy data were required for the registration of both AF36 and NRRL 21882. If the submitted studies supporting health and ecological effects and efficacy in any way suggest that there is some potential for adverse effects, the Agency recommends measures to reduce those risks. For example, the Agency may recommend that there be no application of the pesticide within 30 days prior to harvest, knowing that the chemical decomposes within 14 days after application.

For food use pesticides, registrants must provide data to support a registration decision under the provisions of the FFDCA as amended by FQPA of 1996. These data are used to support claims that residues of the pesticide on treated commodities are safe, and either result in a numerical tolerance or can be exempt from the requirement of a food tolerance. There must be a reasonable certainty that no harm will result from the combined exposure to the expected residues of the pesticide (including dietary and drinking water) and to any cumulative effects from other pesticides with the same mechanism of toxicity. In addition, data or information must show that there are no undue adverse effects posed by non-dietary (i.e. non-occupational dermal, inhalation) and residential exposure. The Agency evaluates these data to establish a tolerance or an exemption from tolerance for residues of the pertinent pesticide on treated commodities.

If these data do not meet all of the Agency requirements for an unconditional safety assessment, a conditional registration may be granted for a specific period of time if use of the pesticide is in the public interest. Additional data are required to be generated within the specified time-frame to provide adequate information so that a decision on the unconditional registration of the pesticide can be made. Conventional wisdom in BPPD, US EPA, is that if Tier II data are required then there may be serious questions about the risks posed by the proposed active ingredient.

Conditional registrations of AF36 and NRRL 21882

For AF36 and NRRL 21882, both registrants provided adequate data to justify conditional registrations. Summaries of Agency evaluations of data submitted in support of these cases can be seen in the technical documents called Biopesticide Registration Action Documents (BRADs) on our website (<http://www.epa.gov/pesticides/biopesticides>) and clicking on Biopesticide Active Ingredients.

Health Effects

Briefly, the data submitted by the registrants demonstrated low toxicity potential for acute oral toxicity/pathogenicity tests in rodents. The pesticides were not considered toxic or pathogenic via pulmonary routes. The intraperitoneal (IP) test was waived for AF36, based on the results of other toxicity/pathogenicity tests and the several years of human exposure during the experimental use permit phase. Unlike AF36, the registrant for NRRL 21882 did submit IP tests in rodents, the results of which indicated potential toxicity or pathogenicity to workers following serious injury. The registrant for this case provided worker exposure of worst-case scenarios to show that such exposure was unlikely from the single, seasonal, low application rates of the granular pesticide.

A state council and six companies reported no adverse hypersensitivity reaction to AF36 during use of the pesticide for 3–6 years. This lack of hypersensitivity

in workers who had been exposed during manufacture, research and application of the pesticide in the research and experimental phases was acceptable to the Agency. Similar arguments were provided from the researchers who had worked with NRRL 21882 for many years in laboratory and field trials. Nevertheless, as with all pesticide registrations, to comply with Section 6(a)(2) registrants are always required to report any hypersensitivity incidents associated with the use of any pesticide.

Acute inhalation data requirements were waived based on the rationales submitted by the registrant. These included the granular nature of the carrier to which the active ingredient adhered, thus reducing inhalation potential. Besides, the active ingredients naturally occur in the environment, with consequent exposure to all. Since the fungi are known as potential dermal and eye irritants, the Agency required, through product labelling, that mixer/loaders and applicators use Personal Protective Equipment (PPE) to mitigate potential risk to workers. Non-occupational and residential exposures are not expected because the pesticide is to be applied at low rates once per year to commercial fields. Exposure was not expected to be greater than that which normally occurs to the naturally occurring microbe, except very soon after the initial application. Both registrants were required to collect and report data to confirm this supposition either as part of the experimental use permit or as a condition of registration.

Ecological Effects

While some strains of *A. flavus* are reported to cause disease in poultry or honeybees, both registrants provided sufficient information in avian intratracheal injection and honeybee studies to satisfy microbial pesticide guideline requirements. Especially interesting was the review of bird habits and habitats for AF36, which indicated that birds do not forage in cotton fields, since they do not eat cottonseeds, and are not likely to be exposed via the oral route of exposure. Data were translated from the mammalian rodent studies submitted in support of health effects to waive data required for mammalian wildlife. Low application rates indicated that the active ingredients are not expected to be incrementally greater than the naturally occurring background levels of the toxic strains. Again, since these strains are considered benign, exposure to them would constitute less of a hazard than exposure to the more toxic strains.

Moreover, the results from the soil- and air-monitoring studies (Cotty, 2001) of AF36 supported rationales to waive data for other non-target organisms such as fish, aquatic vertebrates and invertebrates. The registrants' data for both AF36 and NRRL 21882 provided clear evidence that these naturally occurring soil inhabitants are likely to be more beneficial than their toxic relatives, which are displaced after application. Reports from published literature and available databases indicate that the pesticides, applied at low rates once per year to either crop, were not likely to pose a hazard to endangered species. The evidence also demonstrated that use of the pesticide did not pose any unreasonable adverse effects to the environment and non-target organisms.

Risk Mitigation Considerations

What are the take-home lessons from this cursory examination of these cases? At a first cut, researchers must try to be objective about their favourite projects. They must successfully market their ideas to stakeholders, growers and those willing to commercialize the proposed pesticide products. In addition, the researchers must take into consideration that they will need to have data to be submitted to US EPA that clearly show that the proposed pesticide does not pose any adverse effects to the US population, particularly infants and children. Consumer acceptability does depend, to some extent, on the assessments made by the US EPA.

An examination of these two cases demonstrates that while there are similarities among pesticides, different approaches may be taken to provide data to support their registration. Some of the rationales to support data waiver requests may have been similar in these two cases. However, US EPA's decision to waive guideline-required data is based on review of sound scientific rationales submitted to support those requests. By far, the most interesting take-home message is that scientific enthusiasm must be supported by sound scientific data. This is more crucial when the active ingredients, though benign, may belong to groups containing more toxic relatives.

US EPA has as its mandate the requirement to consider what is important to maintain a safe food supply for the nation. Regulators cannot be swayed by the enthusiasm of researchers and entrepreneurs, nor do they operate in a black box. They have to consider all aspects of a case and try to separate fact from fiction, to sift perceived realities from what is actually realistic. More clearly, they have to discern what the scientific evidence supports and what makes good common sense for both growers and consumers. Only the future and the market can tell whether pesticide products are successful and efficacious.

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29 Postharvest Biocontrol: New Concepts and Applications

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Overview: Biological control of postharvest products has great potential because postharvest environmental parameters such as temperature and humidity can be rigidly controlled to suit the needs of the biocontrol agent. Also, harvested commodities offer a concentrated target for the application of biocontrol agents. In this chapter we provide a personal account of the driving force behind the research and the people that were instrumental in developing postharvest biocontrol technology, the commercialization of new products and the discovery of new science and technologies to optimize the efficacy of the biocontrol process.

In the Beginning

In their book on biological control, Cook and Baker (1983) provided only one example of the biocontrol of postharvest disease of a fruit or vegetable. This was research by Tronsmo and Dennis (1977) in which *Trichoderma* was used to control *Botrytis* rot of strawberry. Subsequently, Wilson and Pusey (1985) presented their ideas on the potential of postharvest biocontrol in a feature article and documented their initial research on using a strain of *Bacillus subtilis* to control brown rot on peach, caused by *Monilinia fructicola*. This seminal work provided the initial ideas and principles that, over the ensuing 20 years, fostered a wealth of research and product development around the world. Numerous reviews have provided an account of the scientific advances that have been made in

postharvest biocontrol, as well as the problems faced in trying to develop a commercial product (Wilson and Wisniewski, 1989, 1994; Droby *et al.*, 2000, 2001; El Ghaouth *et al.*, 2004). While the reader is referred to these reviews and the primary research articles cited within these reports for details on the science of postharvest biocontrol, the present contribution will attempt to provide a personal account of the driving force behind this research and the people that were instrumental in our programme to develop postharvest biocontrol technology, products and science.

While the basic rationale underlying our research efforts was to reduce the use of synthetic chemicals on harvested commodities, our motivation was strengthened by a report by the US National Research Council (NRC) (1987) that stated, 'As a class, fungicides present special difficulties because nine oncogenic compounds account for about 90% of all fungicide sales.' This report indicated that fungicides constitute 60% of oncogenic risk among all pesticides. Furthermore, it concluded that loss of the use of these chemicals would have an adverse economic impact on the production of some crops because of a lack of viable alternatives. This heightened concerns about impending problems associated with the potential decertification of some of the fungicides used to manage post-harvest disease. The potential health risk associated with fungicides was further highlighted by a subsequent report by NRC (US National Research Council, 1993), which documented the increased vulnerability of children to synthetic pesticides. Lastly, reports of the development of resistance to fungicides also helped to establish an urgent need to develop new, effective alternatives for managing postharvest diseases.

Although, at the time, biological control as an approach to managing plant disease did not have any major commercial success stories, we felt that the use of biological control agents in a postharvest environment held special promise. One of the major problems in applying biocontrol agents in the field is that environmental conditions can profoundly affect their survival and effectiveness. In the postharvest environment, parameters such as temperature and humidity are rigidly controlled and can be taken into account when selecting a suitable biocontrol agent. Also, harvested commodities present a more concentrated target for the application of biocontrol agents. The regulated environment, the ability to target the application of the biocontrol agent, and the high value of the harvested commodity together suggested that the use of biocontrol agents to manage post-harvest disease would have an excellent chance of success.

In 1984, it was found that a strain (B-3) of *B. subtilis* was able to control brown rot of peaches caused by *M. fructicola* (Pusey and Wilson, 1984) and the organism was patented. However, it was later determined that the main mode of action of B-3 in controlling brown rot was the production of the antibiotic, iturin. It was felt that there would be resistance to the application of an antibiotic-producing microorganism on food, and commercialization of B-3 was not pursued, even though in pilot tests it demonstrated control of brown rot comparable to synthetic fungicides (Pusey *et al.*, 1988). Interestingly, from a commercial and registration standpoint, this viewpoint may not have been valid as several bio-control products have been developed that utilize antibiotic-producing strains of *B. subtilis*.

The First Generation of Yeast Biocontrol Products

Although the postharvest environment may be especially favourable for the development of biocontrol products, a considerable investment of time and money is required to establish whether a particular organism has commercial potential. The characteristics of an ideal antagonist have been outlined in Wilson and Wisniewski (1989) and are summarized in Table 29.1. While some of these may be obvious, they deserve special consideration prior to committing substantial amounts of research personnel and monies to a project.

After the experience with B-3, two main criteria were considered paramount when we entered into the next phase of the project. First, that we wanted to identify yeast antagonists and, secondly, that the mode of action should not rely on the production of antibiotics by the antagonist. This led to the development of a selection strategy that was later adopted by postharvest biocontrol programmes around the world to identify suitable yeast antagonists (Wilson *et al.*, 1993). Rather than *in vitro* screening of organisms in Petri plates, which favoured the identification of antibiotic-producing organisms, our method involved placing washing fluids obtained from the surface of fruit into fruit wounds that were subsequently inoculated with a rot pathogen. Organisms were then isolated from the surface of wounds that did not develop infections. These were plated out and isolated. Yeasts were identified; pure cultures of potential antagonists were produced; and then each organism was screened individually in fruit wounds to assess its potential as a biocontrol agent. This method identified a number of antagonists that were studied more intensely and measured against the criteria presented in Table 29.1.

Essential to the success of establishing the science of postharvest biocontrol and developing commercial products was the collaborative relationship that was

Table 29.1. Characteristics of an ‘ideal antagonist’ for the postharvest environment.

Genetically stable
Effective at low concentrations
Not fastidious in its nutrient requirements
Ability to survive adverse environmental conditions (including low temperature and controlled atmosphere storage)
Effective against a wide range of pathogens on a variety of fruits and vegetables
Amenable to production on an inexpensive growth medium
Amenable to a formulation with a long shelf-life
Easy to dispense
Does not produce metabolites that are deleterious to human health
Resistant to pesticides
Compatible with commercial processing procedures
Does not grow at 37°C and is not associated with infections in humans
Non-pathogenic to host commodity

formed between us at the USDA-ARS lab (M.W. and C.W.) and scientists working with ARO at the Volcani Center in Israel (E.C. and S.D.). This collaboration began in 1985 and continues to this day. Much of this highly productive collaboration has been funded through the US-Israel Bi-national Agricultural Research Development Fund (BARD).

In the early years of the collaboration, several yeast antagonists were identified that had commercial potential. Our first yeast antagonist, strain US-7 of *Candida guilliermondii*, was originally misidentified as *Debaryomyces hansenii*. This caused some confusion in the patenting process and emphasized the need to have at least two conforming identifications by reputable yeast taxonomic services. It also emphasized the weakness of using physiological tests as the basis for making taxonomic determinations. Using the criteria outlined in Table 29.1, the decision was made, however, to abandon the commercialization of US-7 because other isolates of *C. guilliermondii* had been reported in the medical literature as pathogenic to humans. This decision was made despite the fact that US-7 showed excellent biological control activity and did not show any pathogenicity in Level I toxicology studies. Instead, we chose to focus on the commercialization of *Candida oleophila* (Strain I-182) based on its superior biocontrol activity and the fact that the species did not grow at 37°C. The use of this organism was also protected by a patent.

Another critical ingredient for achieving the goal of a commercial product was the relationship that was developed with a small venture-capital company, Ecogen. This was a US-based company, with a subsidiary in Israel, interested in biological control products. It was the relationship with Ecogen that provided the bridge between theory and practice. They were able to develop a formulated product, based on growing I-182 on a low-cost substrate of industrial by-products, which had an un-refrigerated shelf-life of over 1 year (Wilson and Wisniewski, 1994). Ecogen also provided critical monetary support for conducting semi-commercial pilot tests on apples and citrus in the USA and Israel, respectively. Funding was provided through Cooperative Research and Development Agreements (CRADAs) with the USDA-ARS and similar agreements with ARO. Semi-commercial packing lines, illustrated in Fig. 29.1, allowed us to conduct large-scale studies and determine the performance of a formulated product under more realistic conditions.

The patent on *C. oleophila* was licensed to Ecogen, who handled the complete registration process with the US Environmental Protection Agency and thus the first yeast-based postharvest biocontrol product was launched under the trade name of Aspire™ beginning in 1995. After registration, commercial evaluation of Aspire™ continued in order to better understand how to adapt the use of the product to different packing-house environments and to different commodities (Droby *et al.*, 1998). This led to continued research on how to enhance the reliability and efficacy of the product and established the foundation for a second generation of postharvest biocontrol products (Droby *et al.*, 2003b; El Ghaouth *et al.*, 2004). It is important to note that a parallel but completely independent programme on postharvest biocontrol focusing on bacterial antagonists was being conducted in the USDA-ARS laboratory during this time by Dr Wojciech Janisiewicz. This effort, in collaboration with the US-based company Ecoscience, led to

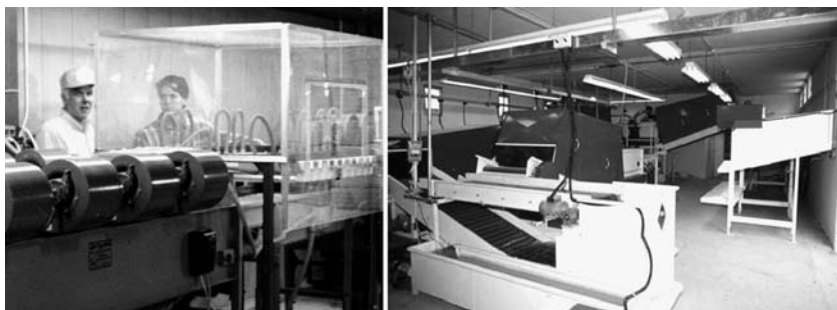


Fig. 29.1. Semi-commercial lines used to evaluate potential antagonists, formulated product and combined treatments such as UV-C light followed by antagonists. Line on left was located at the USDA-ARS facility in Kearneysville, West Virginia and the line on the right was located at the ARO, Volcani Center in Bet Dagan, Israel.

development of Bio-Save™, based on an isolate of *Pseudomonas syringae*. Readers are referred to Janisiewicz (1998) for the details on this effort.

The Science of Postharvest Biocontrol

A short review of the fundamental research we conducted related to postharvest biocontrol is presented because it played a key role in defining and shaping the direction of our programme. Our studies led to the development of key concepts, an expanded view of biocontrol, and greatly influenced the development of a second generation of postharvest biocontrol products.

A main concern was to better understand the features of an organism that made it a good biocontrol agent. In other words, what was the mechanism of action responsible for biocontrol activity? While early studies indicated that nutrient competition and the fast growth rate of our antagonists played a major role in biocontrol activity, subsequent studies indicated a much more complex interaction between the antagonist, pathogen and commodity (Wilson and Wisniewski, 1994). Two novel discoveries were the ability of the yeast to form a biofilm (Fig. 29.2) and, as illustrated in Fig. 29.3, the ability of some yeast antagonists to adhere to and parasitize pathogen hyphae (Wisniewski *et al.*, 1991). The latter report was recognized as the first reported instance of the ability of a yeast to parasitize a higher fungus. Other key factors that appeared to play a role in the efficacy of our yeast antagonists were the production of lytic enzymes by the yeast (Bar-Shimon *et al.*, 2004) and their ability to tolerate high levels of salts (Wisniewski *et al.*, 1995). The induction of resistance responses in the fruit by application of the antagonists within a wound or on the fruit surface was also a novel discovery (Wilson and Wisniewski, 1994; Droby *et al.*, 2002; El Ghaouth *et al.*, 2003).

More recently, we have used molecular approaches to examine the role of glucanases in biocontrol activity of the yeast *C. oleophila* (Yehuda *et al.*, 2003) and to enhance biocontrol activity by overexpression of antimicrobial peptides (Wisniewski *et al.*, 2003).

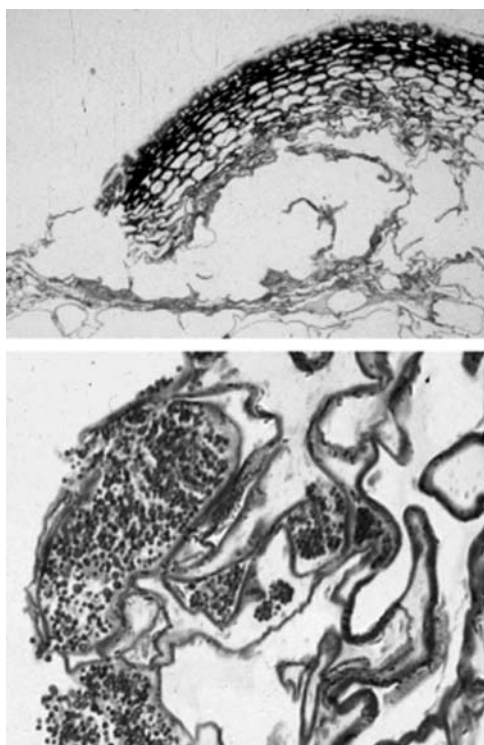


Fig. 29.2. Ability of yeast antagonists to form a 'biofilm' in wound sites of fruit. Top picture shows *Candida oleophila* forming a film along surface of wound in apple (100x). Bottom figure is an enlargement of top figure and highlights the large number of cells and yeast extracellular matrix involved in forming a biofilm.

Out of the Frying Pan and Into the Fire

By early 2000, there were three postharvest biological products available on the market: Aspire™ (limited to the USA and Israel), Bio-Save™ (limited to the USA) and YieldPlus™ (limited to South Africa). In spite of all the published fundamental and applied research on postharvest biocontrol, the commercial use of these products was, and remains, limited and accounts for only a very small fraction of the potential market. Despite this, however, it is commonly recognized that this area of biocontrol has tremendous potential for economic success. As discussed in recent reviews (Droby *et al.*, 2003a; El Ghaouth *et al.*, 2004), the main shortcoming with the use of postharvest biocontrol products has been inconsistency in performance, especially when used as a stand-alone product to replace synthetic fungicides. A second problem with the current generation of products is their inability to control previously established and latent infections. The reasons for these shortcomings have been reviewed by Droby *et al.* (2003a). In brief, the following factors can dramatically affect the viability and performance of postharvest biocontrol agents: (i) fermentation and formulation practices; (ii) the method by which the product is delivered to the commodity; (iii) inoculum pressure; and (iv) the physiological status of the fruit. Additionally, the strategy used to identify potential antagonists favoured the selection of organisms that exhibited protective rather than eradication activity.

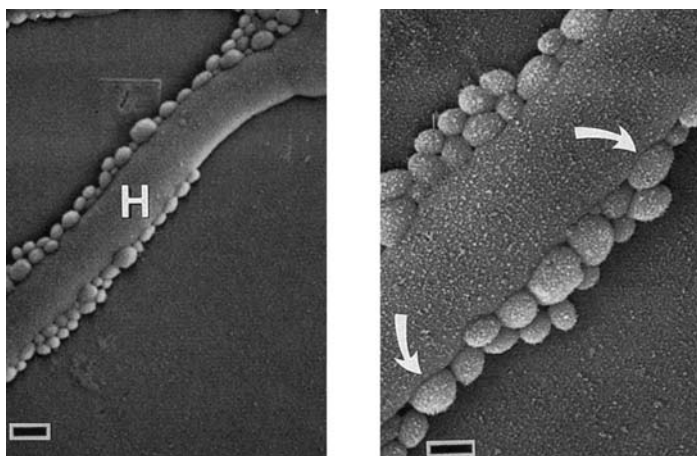


Fig. 29.3. Attachment of *Pichia guilliermondii* (Strain US-7) to hyphae of *Botrytis cinerea*. Note concave appearance of hyphal wall in right-hand picture. Scale = 2.5 μ m.

Throughout the course of developing Aspire™ considerable research went into finding methods to enhance the reliability and efficacy of the product and other selected antagonists as well. In particular, it was our intention to find additives or physical control methods that would act synergistically with our antagonist. Initially, this involved combining the product with a low level of postharvest fungicide (Droby *et al.*, 1998) or 1–2% salt solutions of calcium chloride or sodium bicarbonate and other additives commonly used in the food industry (Droby *et al.*, 2003b). It was also reported that physical treatments such as hot air, curing, hot-water brushing and combinations of the above with pressure infiltration of calcium could also increase the efficacy of antagonists (reviewed by Droby *et al.*, 2003a). In collaborative research with one of us (C.S.), a pioneer in the use of low-dose UV-C light as a means of inducing host resistance to decay in harvested commodities, we also demonstrated that this approach could enhance the performance of yeast antagonists (Stevens *et al.*, 1997). Combining antagonists with a sugar analogue (2-deoxy-D-glucose) was also suggested as an approach to increase efficacy (Janisiewicz, 1994; El Ghauth *et al.*, 2000). However, due to the high cost of the sugar analogue this aspect was not pursued commercially.

The Second Generation of Yeast Biocontrol Products

During the course of our research it was realized by one of us (C.W.) that if postharvest biocontrol was going to be commercially successful a broader concept of biological control would be needed. Plant pathologists have adopted the entomologists' definition of biocontrol, which involves the control of one organism with another organism. But, a plant disease is not an organism. It is a process. Therefore, we have defined the biological control of a plant disease as

'control of a plant disease by a biological process or the product of a biological process'. (See also discussion by Cook, Chapter 44 this volume.)

Using this broader definition of the biological control of plant diseases, a number of avenues become available for developing effective, commercially successful biological control products and practices: (i) the classical idea of using an antagonist; (ii) innate or induced resistance, which is a biological process; and (iii) natural antimicrobials, which are the product of a biological process. While some of these approaches are being pursued by us and others (as outlined above) without commitment to a formal paradigm, it is important to conceptualize the paradigms that drive scientific research, in order to overcome limitations and expand possibilities. This new paradigm of biological control was the primary concept that we used to develop a second generation of postharvest, biocontrol products.

In 1992, a new employee (A.G.) from Laval University in Quebec, Canada arrived at the USDA-ARS laboratory in Kearneysville. He brought with him a wealth of knowledge and experience on the use of chitosan as an antimicrobial compound. During his 10-year tenure here he was instrumental in the development of a second-generation postharvest biocontrol product and documenting the role of induced resistance in the mode of action of our yeast antagonists.

The main objective in developing a new product was to address the poor ability of Aspire™, and other postharvest biocontrol products, to control pre-established and latent infections. We hoped to overcome this by using a combination of natural products along with a yeast antagonist. We also decided at that time to focus on a new yeast antagonist in order to enhance patent opportunities and attract new industrial partners. These research efforts led to the development of two new products, whose main components consisted of the yeast antagonist *Candida saitoana* and a derivative of either chitosan (Biocoat) or lysozyme (Biocure). Both of the compounds had been tested worldwide and shown to have strong eradicated activity (Fig. 29.4). The two commercial products also contain other additives such as sodium bicarbonate. The additives were found to enhance control efficacy to levels equivalent to that found with available postharvest fungicides. Patents have been issued to cover this technology (El Ghaouth and Wilson, 2002; Wilson and El Ghaouth, 2002). While this research was initially conducted under a CRADA with American Cyanamid and then MicroFlo (a subsidiary of BASF), the technology has now been licensed to Inova Technologies and is awaiting registration by the US Environmental Protection Agency.

A more recent product (developed by S.D.) has taken the approach of preventing postharvest decay by application of a yeast biocontrol agent to flowers and fruit in the field, several times throughout the growing period. This approach also addresses the problems of pre-established and/or latent infections. The product is based on the use of a heat-tolerant strain of *Metschnikowia fructicola* and is marketed under the name ProYeast-ST and ProYeast-ORG in Israel by the company AgroGreen. It has been shown to be effective against rots caused by *Botrytis*, *Penicillium*, *Rhizopus* and *Aspergillus* on strawberries (Karabulut *et al.*, 2004), grapes and citrus.

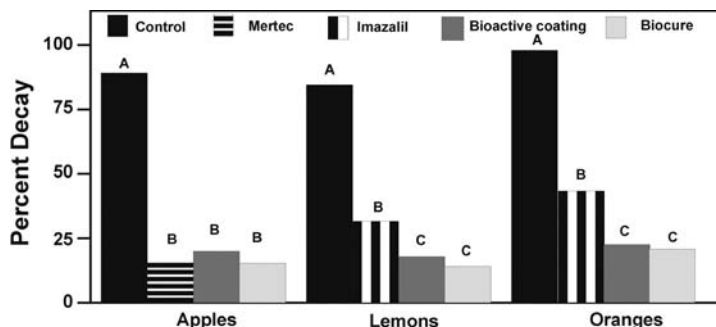


Fig. 29.4. Control of pre-established infections on apples, lemons and oranges by Biocoat (Bioactive coating consisting of the yeast *Candida saitoana*, chitosan salt and other additives) and Biocure (*Candida saitoana*, lysozyme and other additives). All fruit were wounded and exposed to decay pathogens 24 h prior to exposure to biocontrol preparation or fungicide. Mertec was the fungicide used on apples, and Imazalil was the fungicide used on citrus. Apples were stored at 18°C for 4 weeks prior to decay assessment while lemons and oranges were stored at 10°C for 28 days prior to decay assessment.

Where We Stand and Where We Go

The past 25 years have seen tremendous growth in the science and practical application of biological control of postharvest diseases. The available literature has expanded from a few publications in the early 1980s to hundreds, if not thousands, by 2005. The number of labs that conduct research in this area has also changed from 2–3 located in the USA and Israel to dozens located throughout the industrial and developing world, and several products have been made available. Our own success and influence in this field of research was a direct result of having a timely idea (i.e. being at the right place at the right time), the strong collaboration between the USDA-ARS and ARO laboratories, and the involvement of industry and their expertise and drive to develop a commercial product. International cooperation with South Africa, Brazil, Australia, Egypt, Italy, New Zealand, Mauritania, Turkey and Uruguay, which took the form of visiting scientists, graduate students and product-evaluation arrangements, also played an important role in fostering our success and prominence. Truly, the small beginnings at the Appalachian Fruit Research Station (USDA-ARS) and the Volcani Center (ARO) blossomed into a worldwide effort.

As indicated, the use of the available postharvest biocontrol products thus far has been rather limited, given the potential market. Some of the reasons for the lack of adoption of these products have been overcome in the ‘second-generation’ products that are, or will soon be, available. The future success of these products will depend on market conditions. Synthetic fungicides have a long history of use, are generally easy to apply, and continue to be highly effective. Growers will only replace chemical pesticides with biologicals if there is a continued demand by consumers for pesticide-free food products. Organically

grown fruit represents a large potential market for use of biological agents, since the use of synthetic fungicides is strictly prohibited. The demand for such produce has seen tremendous growth in the last decade and this does not seem to be slowing down. Importantly, new biological postharvest products must be adaptable and effective as stand-alone products, without the need for additional inputs if they are to be competitive with synthetic fungicides. Postharvest biologicals must also begin to address problems of decay management in commodities where postharvest disease is harder to control, such as stone fruits and berries. Lastly, the huge potential of providing extended decay control to the consumer, prior to and after commodity purchase, through the use of antimicrobials in modified and intelligent packaging should be recognized.

The greatest hope for a biological approach (using a broad definition of biological control) lies in a further understanding of the mechanism(s) of action of microbial antagonists and natural products, innate and induced resistance in the host, and the biology of decay pathogens. It is expected that this knowledge will lead to new, innovative approaches for controlling decay in harvested commodities and presents the best hope for the future of the biological control of postharvest disease.

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30 Development of the Mycoherbicide, BioMal®

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Overview: *Colletotrichum gloeosporioides* f. sp. *malvae* was discovered by fortuitous observation as blight on seedlings of round-leaved mallow (*Malva pusilla*), a serious weed pest in prairie agriculture. This chapter describes the history of bringing this fungus to market as a post-emergent bioherbicide, trade named BioMal®.

What Are Mycoherbicides and Why Do We Need Them

Crop pests such as weeds, insects and plant diseases contribute significantly to crop yield loss in agricultural production. Globally, over \$25 billion was spent on chemical pesticides in 2002, with herbicides accounting for almost 50% of all pesticide sales (CropLife International, 2002). Farmers use a variety of weed control methods including cultural, chemical, mechanical, biological and genetic practices, but the majority of current cropping practices continue to rely heavily on chemical pesticides. This has led to the development of herbicide-resistant weeds, with more than 178 weed species (107 dicots and 71 monocots) reported to have resistance to chemical herbicides (Heap, 2005). Increasing public concern over environmental issues related to chemical pesticide residues in food, soil and water, the de-registration or phasing out of a number of older chemicals, and the restriction or banning of chemical pesticides in urban municipalities underscore the need for reduced-risk pest control products. Biological weed control exploits plant pathogenic microorganisms for weed management. Such products provide one more tool to mitigate or delay the development of herbicide-resistant weeds, thereby extending the effectiveness of chemical herbicides while also reducing the dependency on such chemicals.

Bioherbicides are typically plant pathogenic fungi or bacteria that infect weeds. They can be artificially mass produced and applied to the foliage or soil in order to cause significant damage, suppression or control of a target weed. When the control agent is a fungus, the control product is labelled as a 'myco'-herbicide.

Successful bioherbicides typically have a narrow host range with no significant effect on non-target plants, particularly the crop or endangered species, have high efficacy (e.g. weed control or mortality), are consistent in their performance in the field and can be easily mass produced and applied with conventional farm equipment. Formulations are designed to rapidly disperse in water and protect the spores from shear forces encountered in conventional spray equipment. Products that cannot be applied at 'practical' rates generally do not become commercial successes. Bioherbicides are not expected to replace chemical herbicides, but can be used as a complementary method in weed management.

Researchers began to explore the potential of plant pathogenic fungi as mycoherbicides over 4 decades ago. Two fungal pathogens were registered and commercialized as mycoherbicides in the USA: Collego[®], a host-specific fungus (*Colletotrichum gloeosporioides* f. sp. *aeschynomene*) for biological control of the weed northern jointvetch, and DeVine[®], a soil-borne pathogenic fungus (*Phytophthora palmivora*) that controls strangervine weed in Florida citrus groves (Boyetchko and Peng, 2004). These initial successes greatly stimulated the interest in mycoherbicides worldwide. Researchers in Canada began to explore the potential of using plant pathogens for biological weed control in the mid-1970s. In 1982, a plant pathogenic fungus was isolated from round-leaved mallow (*Malva pusilla*) (Fig. 30.1) at the Agriculture Canada Research Centre (AAFC) in Regina, Saskatchewan. It was recognized to have great potential as a mycoherbicide.



Fig. 30.1. Round-leaved mallow (*Malva pusilla*) showing long branches and prolific seed production.

Early Discovery, Evaluation and Development of BioMal®

The discovery in 1982 of *Colletotrichum gloeosporioides* f. sp. *malvae* (Cgm) resulted from a fortuitous observation of seedling blight on round-leaved mallow being grown in growth cabinets for use in tests of chemical herbicide efficacy (Mortensen, 1988; Makowski and Mortensen, 1992). The following year, the fungus was found to occur naturally on round-leaved mallow in a number of fields throughout Saskatchewan and Manitoba, with epidemic levels of the disease developing late in the growing season. Since differences in pathogenicity were not observed with the Cgm isolates on round-leaved mallow, the isolate originally obtained from *M. pusilla* seed was advanced for development as the mycoherbicide BioMal® (ATCC 20767) (Fig. 30.2). Round-leaved mallow is a weed found in farmyards, gardens and waste areas, and it causes significant yield losses in crops such as flax and lentil, which are not highly competitive crops (Makowski and Mortensen, 1992; Mortensen and Bailey, 2002). Yield losses in wheat as high as 20–30% have been reported. The weed produces a long tap root and extensive amounts of seed. At the time of discovery of Cgm, some herbicides were known to provide good control of young seedlings, but they did not provide adequate or consistent control of older plants. Cultivation, mowing and grazing were only partially effective owing to rapid recovery and regrowth of the weed.

The disease causes anthracnose symptoms on stems and petioles (Mortensen, 1988) (Fig. 30.3). The dark sunken lesions have grey centres with black margins, which eventually coalesce and girdle the stem, resulting in wilting of the plant. Under moist conditions, sticky masses of conidia (i.e. spores) are

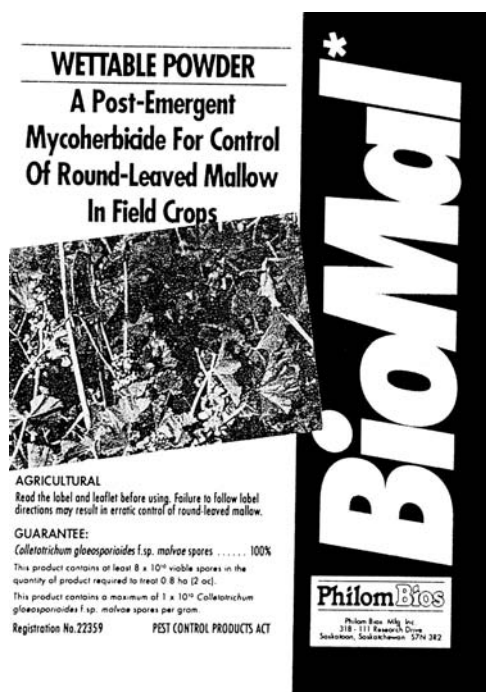


Fig. 30.2. BioMal® product information label.

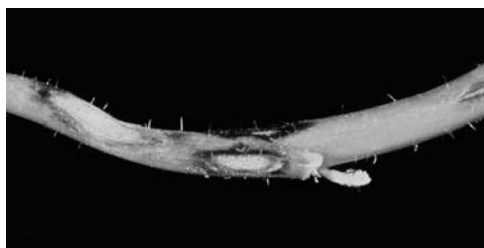


Fig. 30.3. Disease symptoms on round-leaved mallow stems caused by *C. gloeosporioides* f.sp. *malvae*. Note the dark sunken lesions with grey centres and black margins.

produced in fruiting bodies known as acervuli on infected plant tissues. The spores are spread by the splashing of rain drops, which create new points of infection on the same plant or on neighbouring plants. There is no known sexual stage for Cgm (Mortensen and Bailey, 2002). The disease cycle is completed by the fungus overwintering on *M. pusilla* stems and other susceptible plant residue (Makowski and Mortensen, 1999).

During the early stages of evaluation, Cgm was considered to be a good mycoherbicide candidate for three reasons: (i) the initial results showed that disease development was restricted to the Malvaceae family with only a few exceptions; (ii) the fungus could be produced on artificial media; and (iii) foliar applications of spore suspensions effectively controlled *M. pusilla* under field conditions (Mortensen, 1988; Makowski, 1993; Mortensen and Bailey, 2002). Initial host range testing was conducted under greenhouse conditions and showed that disease symptoms were most severe on *M. pusilla*, *Malva parviflora*, *Malva alcea* var. *fastigiata*, *Malva moschata* and *Abutilon theophrasti* (Table 30.1). Other mallow species had either no symptoms or restricted lesion development, where the plants outgrew the disease. Only two non-target species from 11 other plant families showed restricted lesion development to Cgm inoculations: *Carthamus tinctorius* (safflower) and *Brassica hirta* (white mustard). All other species remained symptom free. As the evaluation progressed, additional data were required for registration to ensure crop safety. Studies were conducted in the field to assess inoculations on nine non-target crops (oilseed rape, flax, lentil, mustard, safflower, sugarbeet, sunflower, wheat, strawberry) for disease development, crop development, biomass and yield (Mortensen and Makowski, 1995, 1997) (Fig. 30.4). There were no adverse effects on plant development or yield for these crops, except for safflower (Table 30.1).

Efficacy, a crucial trait for any mycoherbicide, was excellent for Cgm right from the beginning. Results from over 20 field trials conducted in different site-years indicated that the mean percent control of round-leaved mallow was 73%, with 13 of the trials showing greater than 90% control, three trials with greater than 60% control, and only four failing to provide control (Mortensen, 1988). The main factors influencing efficacy were inoculum concentration, temperature and dew period (i.e. duration of leaf wetness) (Makowski, 1993). Cgm caused disease at all weed growth stages, although young seedlings were less susceptible than older plants. The optimal spore concentration needed to provide maximum control was $2-4 \times 10^6$ spores/ml. Lower concentrations resulted in slower disease development, but under ideal moisture conditions secondary

Table 30.1. Addressing issues of environmental toxicology, environmental fate, and food and feed residues through studies on pathogenicity, crop tolerance and pathogen recovery with *C. gloeosporioides* f.sp. *malvae* on target and non-target plants. (From Mortensen, 1988; Mortensen and Makowski, 1995, 1997; Makowski and Mortensen, 1999.)

Target species in Malvaceae		Non-target crop species			
Scientific/ common name	Patho- genicity	Scientific/ common name	Patho- genicity	Tolerance	Recovery
<i>Malva pusilla</i> Round-leaved mallow	8.3*	<i>Triticum aestivum</i> Wheat	0.0	100	0.1
<i>M. moschata</i> Musk mallow	7.0*	<i>Linum usitatissimum</i> Flax	0.0	100	0.1
<i>M. alcea</i> var. <i>fastigiata</i> European mallow	6.7*	<i>Brassica</i> sp. Oilseed rape	0.0	100	0.5
<i>M. parviflora</i> Small-flowered mallow	6.3*	<i>Brassica/ Sinapis</i> sp. Mustards	0.0	100	0.2
<i>Abutilon theophrasti</i> Velvetleaf	6.3*	<i>Helianthus annuus</i> Sunflower	3.0*	100	0.1
<i>M. neglecta</i> Common mallow	4.3	<i>Beta vulgaris</i> Sugarbeet	1.8	100	0.0
<i>Malope trifida</i> Mallow (ornamental)	4.0	<i>Carthamus tinctorius</i> Safflower	4.7*	71*	6.6*
<i>M. sylvestris</i> High mallow	2.0	<i>Abelmoschus esculentus</i> Okra	0.0	100	Not tested
<i>Hibiscus</i> sp.	0.0	<i>Lens culinaris</i> Lentil	1.2	100	0.0
<i>Althaea rosea</i> Hollyhock	0.0	<i>Fragaria</i> sp. Strawberry	0.0	100	3.3 – leaves 6.0 – berries

Pathogenicity = disease rating 14–20 days after inoculation using a 0–9 scale, where 0 is no symptoms and 9 is > 90% dead plant material; for target species a rating of 5 was considered significant; for non-target species comparisons were made with untreated controls but data not shown.

Tolerance = crop biomass expressed as a % of the untreated control.

Recovery = % of plant parts with Cgm relative to the total plated at 2 weeks after inoculation; recovery from untreated controls was used for comparative purposes but data not shown.

*Significantly different from the untreated control at $P < 0.05$ using an LSD test.



Fig. 30.4. Control of round-leaved mallow in strawberry plots treated with *C. gloeosporioides* f.sp. *malvae* (Cgm). Dead plants are the weed infected with the Cgm.

infection cycles produced more inoculum, which enhanced weed control later in the season. For infection to occur, temperatures between 15 and 25°C and dew periods of 16–48 h were required to achieve 80% control of round-leaved mallow within 1–3 weeks after application (Makowski, 1993). Shorter, repetitive dew periods could compensate for a single, long dew period; however, disease development was best when the dew came immediately after inoculation. Disease on velvetleaf was evident with an application of Cgm, but weed control was less effective and resulted in less than 80% kill.

Thoughts about commercializing the technology were initiated early in the evaluation process and an industry partner was sought. In 1985, an agreement for the rights to commercialize was signed between AAFC and Philom Bios Inc. The company initiated patent protection under AAFC's name for using Cgm as a mycoherbicide in Canada (patent issued November 1990) and Europe (issued December 1989, individual national patents were not pursued). A joint patent between AAFC and Philom Bios was filed in the USA for the use of Cgm based on the manufacturing process developed by Philom Bios and it was issued March 1994. A data package was submitted in 1987 to the Pesticides Directorate (now known as the Pest Management Regulatory Agency) for registration in Canada. Finally, on 16 January 1992, Cgm was registered in Canada under the trade name BioMal® for use as a post-emergent bioherbicide on wheat, oats, buckwheat, mustard, sunflower, soybeans, barley, rye, oilseed rape, flax, lentil and sugarbeet.

Challenges in the Commercial Development and Registration

When the initial data registration package was prepared and submitted to the Canadian regulatory agency, the guidelines and protocols for registration of microbial pest control products in Canada did not exist (Makowski and Mortensen, 1992; Cross and Polonenko, 1996). Many of the data requirements, particularly the human-health safety testing, environmental toxicology and residue/persistence

data, were loosely based on those requirements for chemical pesticides. Through consultations between AAFC, Health and Welfare Canada and Environment Canada, additional tests that addressed these data requirements were generated and also met with approval from US EPA for microbial product registration (Mortensen and Bailey, 2002).

Characterization, biocontrol potential and non-target effects of Cgm were documented, usually through studies conducted under controlled environment conditions and in small-plot field trials. However, concerns over latent infections occurring on non-target crops and questions about infected but symptomless residue contributing to the inoculum load and increasing selection pressures in the environment caused the regulatory agency to request more testing. Makowski and Mortensen (1998) inoculated the field crops to study the infection process and found few mature appressoria (i.e. infection structures) and minimal host penetration. Even though Cgm was recovered from the inoculated crop plants, the frequency of recovery declined with time, regardless of host association, such that more than 2 weeks after inoculation, the recovery of Cgm from plant tissues was only obtained from safflower (Makowski and Mortensen, 1999). These studies alleviated concerns about increasing systemic latent infections from the release of millions of spores on non-target plants, increasing and maintaining higher than normal levels of inoculum in the environment, and posing a risk to utilizing the crops for food and feed.

Although efficacy of Cgm in the field was demonstrated and its potential as a mycoherbicide clearly established, the complexities of the technical hurdles involved in the product development were underestimated. BioMal® was mass produced in submerged (liquid) fermentation, and the discovery of an enzyme that was involved in the onset of sporulation was an important step towards determining the most cost-effective means of production of the fungus (Cunningham and Kuiak, 1989). The product itself was formulated as a wettable powder, which consisted of spores of Cgm that could be stored at room temperature for extended periods prior to use. However, all efficacy trials conducted in the greenhouse and field utilized only spores suspended in water without the aid of adjuvants in the spray application, which could protect spores from desiccation and promote better spray application coverage on the leaf and stem surfaces. Furthermore, the spore suspensions were sprayed until runoff or with high water volumes, which are impractical for farmers to apply. Under controlled environment conditions, optimal dew periods (16–48 h) were provided to promote infection and disease development, yet this mycoherbicide was registered for use in the Canadian prairies, where the dew period is frequently less than 8 h. Therefore, it was critical that formulation additives be included to promote leaf wetness and encourage spore germination and infection. In laboratory studies, Zhang *et al.* (2003) reported that the adjuvants Tween 40 and 80 promoted conidial germination of Cgm, whereas Tergitol was detrimental and Tween 20, sorbitol and gelatin were neutral. For formulation development and application strategy there is a need for much more basic understanding of the mycoherbicide–weed interaction.

In 1994 Philom Bios halted production and sale of BioMal® to western Canadian crop producers because 'the market potential was simply too small to justify further commercialization costs and production expenses' (Cross and

Polonenko, 1996). This young industry was taught three tough lessons from their efforts to commercialize BioMal®. One, do not underestimate the complexities of growing microorganisms and the difficulties involved in scale-up fermentation and formulation. With Cgm both aspects proved to be challenging and consequently economically unfeasible and time consuming. Two, ensure that you have sound market research. In re-evaluating the potential market size for BioMal® the company discovered that the area infested with round-leaved mallow was smaller than earlier predictions. This made the cost of production of the mycoherbicide for the potential acreage unacceptable to crop producers. Three, the competition will protect their markets. During the time that was required to develop mass production and formulation protocols and for registration, competitors introduced three chemical herbicides (i.e. Estaprop, Dyvel DS and Refine Extra) for control of round-leaved mallow (Cross and Polonenko, 1996).

In 1998 a new licensing agreement was signed with Encore Technologies (Carlson Business Centre, Minnetonka, Minnesota 55305, USA) to pursue re-registration and commercialization of Cgm through US EPA under the name Mallet WP. The company wanted to expand the market for this product to small-flowered mallow and improve its field performance under broader environmental conditions (cool temperatures and humid conditions). Continued difficulties in manufacturing a consistent product were encountered and the company decided to not pursue commercialization of Mallet WP.

What is the Future of Mycoherbicides?

BioMal® was the first mycoherbicide product to receive registration approval in Canada but, to this day, it is not commercially available to producers. BioMal®, however, served a larger purpose for the Canadian regulatory body, as they transformed sketchy guidelines for registration of microbial pest control products and used our experience to develop the first set of data requirements for registration of biopesticides. In hindsight, we could have made better decisions as to the potential for commercialization of BioMal® by earlier validation of the scale-up for production and by testing the product in large-scale field studies (Cross and Polonenko, 1996). Not doing this failed to reveal the potential problems that we were to encounter.

Cost-effective fermentation and formulation technology for mycoherbicide product development are invariably linked to market size and crop value. A cost-effective and reliable fermentation process is needed to generate optimal yields of stable, highly effective propagules (active ingredient). This needs to be combined with a user-friendly formulation technology which stabilizes the active ingredient for extended shelf-life and delivery for spray application. While BioMal® could be stored for extensive periods as a wettable powder, formulation additives that could reduce the dew period requirements present in the normal Canadian prairie conditions were not found.

These early mycoherbicides proved that the complexities for product development and commercialization are easily underestimated. Many of the factors that curtailed the marketing of this product, such as scale-up, mass production,

formulation and market size, were not understood. While early-stage development of such products is usually undertaken by public research institutions, the major investment and product development are often carried out by small to medium-sized companies, which generally lack the capital to invest in the product development. It may be prudent to evaluate the stage at which an industry partner becomes involved rather than risk handing off the technology prematurely.

So what is the future of mycoherbicides such as BioMal®? Philom Bios Inc. continues to maintain the patent for BioMal®, and future commercialization opportunities and other markets for the product are being analysed. Given the limited market size, the key questions to the company might be: (i) how much would it cost to optimize the product and bring it to market; and (ii) are there added benefits to bringing BioMal® into the market that are not supplied by other products. Initiatives from AAFC are probably required to adequately address the product's technical shortcomings before a marketing decision can be reached. Advancements in a variety of mass-production and downstream-processing strategies and formulation technologies that address critical issues of product stability, shelf-life and environmental challenges to reduce dew period requirements and improve field performance have been made. Based on the past experience with BioMal®, we need to develop a coherent strategy for development of future mycoherbicide (and bioherbicide) products that sufficiently addresses critical technical challenges in partnership with industry prior to a decision on commercialization.

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31

Development of *Chondrostereum purpureum* as a Mycoherbicide for Deciduous Brush Control

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Overview: The fungus *Chondrostereum purpureum* was isolated from a canker on a diseased apple tree in British Columbia. Extensive laboratory and field trials demonstrated that *C. purpureum* effectively prevents sprouting of cut stumps of deciduous, but not coniferous, trees by colonizing and decaying their stumps. This chapter focuses on the research activities that led to the successful development of *C. purpureum* as a biocontrol agent of woody deciduous weeds in reforestation sites and other corridors where brush control is required.

Discovery

When selecting potential candidates for development as a mycoherbicide it is essential to consider the entire life history of the organisms under consideration. The early identification of a candidate for the control of deciduous brush was made by Dr Ron Wall of the Canadian Forest Service's Pacific Forest Research Center. He isolated what eventually became our lead isolate for the development of the fungus *Chondrostereum purpureum*. This organism is now registered in both Canada and the USA as a biocontrol for woody deciduous brush in forest regeneration sites and industrial corridors for hydroelectric power. This bioherbicide provides an attractive alternative to the use of chemical herbicides and provides one more tool for an integrated weed management programme in North American forests and utility rights-of-way.

The story begins with the collection of an isolate of *C. purpureum* from a canker on a diseased apple tree in Saanichton, British Columbia in 1989. In 1994 Dr Wall inoculated a red alder (*Alnus rubra*) with this isolate at a site near Duncan, British Columbia and re-isolated the fungus from the resulting canker 1 year later. Unbeknownst to Ron this turned out to be the historic moment that led us on a journey to the full registration of this fungus as a forest biocontrol agent in 2005.

Life History of *C. purpureum*

The fungus *C. purpureum* is extremely widespread and occurs throughout the temperate regions of the northern and southern hemispheres, as attested by our very large collection of isolates. The only reported ecological niche for *C. purpureum* is in the xylem of living trees and shrubs or in trees and shrubs that have died within the past 2 years (Duncan and Lombard, 1965; Rayner, 1977). It prefers broadleaved trees as hosts (Dicotyledonae) and does not generally infect conifers, making it particularly useful in reforestation sites. The fungus survives as mycelia in infected trees and is able to replicate and disseminate in the environment through the production of basidiospores from fertile basidiocarps, which it produces on recently killed woody tissues (Dye, 1974). The airborne basidiospores are short lived (<5 h), probably due to sensitivity to desiccation and ultraviolet light (Grosclaude, 1969). However, if they reach a moist wounded surface, they may germinate, and the resulting hyphae can penetrate and ramify through the plant tissues inter- and intracellularly (Spiers and Hopcroft, 1988).

One of the most significant features of the isolate originally collected by Dr Wall is that it proved to be a surprisingly weak plant pathogen. This was shown in bioassays we developed to measure the relative virulence of our extensive collection of *C. purpureum* isolates as a means to rank their potential as biocontrol agents. In the early bioassay work we used mostly black cottonwood (*Populus balsamifera* L. ssp. *trichocarpa*) and red alder (*A. rubra*) as host plants. Stem cuttings of black cottonwood were inoculated with different isolates of the fungus and either incubated in moist chambers or planted in rooting medium. Virulence was measured as the time interval between inoculation and mortality of the cutting. This proved to be an inconsistent predictor for relative virulence and it was found that greenhouse trials provided a much better ranking of isolates. Seedlings of red alder were grown in 5-litre pots and inoculated when they were 1 year old and about 1 m in height and 2 cm in diameter. In the spring, shortly before bud break, freshly cut stumps about 10 cm high were inoculated with the fungus. Dieback, as measured by visible discoloration of the stumps, was observed within 1 month and was significantly greater than the control for most isolates. Of this lot the original isolate still remained one of the best performers but other isolates were equally efficacious. The best indicator of relative efficacy, however, was revealed only in full field trials. We thus recommend moving to actual deployment of a formulated product for development purposes as soon as possible.

Selection of the Best Candidate Isolate

Researchers intuitively seek the most aggressive isolates for development of biocontrol agents. However, for some applications selection of a weak pathogen that can be made more effective by application technologies or by taking advantage of conditions created during the cultural practices may be a better strategy. The weak pathogenicity of *C. purpureum* was, in fact, a great advantage to us, as it provided a level of security that we would do no harm when we released this

plant pathogenic fungus into the field. Initially, this was a serious concern, impeding the development of *C. purpureum* as a biocontrol agent. The fungus is known to have a broad host range, and massive deployment could theoretically have resulted in epiphytotics (disease epidemics in plants). Most weed biocontrol agents that have been developed to date have a narrow host range, often limited to a specific species or cultivar of a target weed. Although *C. purpureum* can attack a broad range of plant species its impact is limited by its life history. For one, it can only invade a host plant through wounds in the xylem. Second, it is a weak pathogen and even when it does infect it merely causes mild sap-streak symptoms in many infected trees. It kills infected trees only when they are severely stressed, such as when the tree is girdled by placing inoculum in contact with the entire cambium (Fig. 31.1). In experiments where only half of the girdled region was inoculated with the fungus, the tree grew new shoots from the side of the stem not inoculated. Single-point infections will result in cankers but



Fig. 31.1. Fruiting structures of *C. purpureum* are commonly found on wounded stems of *A. rubra* (red alder) and other deciduous trees. The stems above the site of infection usually die off while those below the site of infection may continue growing.

rarely in mortality. Therefore, because of this built-in safety feature we recommend that the entire cambium of a stump be treated in order to obtain full weed control. The fungus is applied as a paste formulation to the cambial layer of target trees following cutting with a chainsaw or brushsaw. Operationally this closely parallels application of the herbicide glyphosate (also as a paste) to cut stems. Hence, there was a high acceptability of using the fungus because it did not require many changes in application technology. Furthermore, despite its broad host range, the non-target species are not likely to be affected unless they are wounded during inoculum dispersal (de Jong *et al.*, 1990).

Environmental Fate and Impact of Release

The possible introduction of rare virulence alleles from the released organism into a local population of the pathogen is a recurring concern in the development of any biological control agent. Since *C. purpureum* is a fairly ubiquitous organism throughout North America we expected that there would already be extensive gene flow between populations. Thus the risk of introducing rare virulence alleles into novel genetic backgrounds would be minimal. *C. purpureum* relies on sexual reproduction for dissemination, and its genetic system promotes outcrossing. It has a tetrapolar mating system, and sexual compatibility studies between single spore isolates suggest the presence of multiple alleles at two mating type loci (A and B), as in the closely related basidiomycete *Schizophyllum commune*. Vegetative incompatibility barriers have not been observed; hence a fertile heterokaryotic mycelium can be formed in all sexually compatible matings. In homokaryotic isolates from widely separated regions, mating would be expected to be 100% successful because the multi-allelic nature of the A and B factors ensure compatibility between isolates (Boidin, 1986).

As with any biocontrol that is applied in an inundative manner there were questions of the general or long-term increase in populations of the fungus beyond normal endemic levels. We therefore developed a series of genetic markers to assess natural variation in local and continental populations and to follow specific isolates following field release. The population structure of *C. purpureum* across Canada was determined for four different genetic markers. Variation was assessed using markers for ribosomal DNA, mitochondrial DNA, randomly amplified polymorphic DNAs (RAPDs) and sequence characterized amplified regions of the genome (SCARS) (Ramsfield *et al.*, 1996, 1999; Gosselin *et al.*, 1999; Becker *et al.*, 2004, 2005). Analysis of the distribution of these markers indicated that there were indeed no barriers to gene flow and that the introduction of rare pathogenicity alleles from isolates used as biological control agents had a very low probability. Taken together these studies indicated that the risk involved in using one isolate as a biological control agent across all ecozones of Canada was relatively low. It was concluded that additional infection due to the release of *C. purpureum* would be of the same order of magnitude or less than infection due to naturally occurring sources of inoculum. This was confirmed by spore-trap data at a field release site in Duncan, British Columbia (Becker *et al.*, 2005).

These same genetic markers were also used to monitor infection of non-target vegetation during and after deployment of the biocontrol. There were no incidences of infection detected of non-targets of either conifer or deciduous brush following the application of the formulated fungus to specific cut stems (Becker *et al.*, 1999). Other non-target species that we were asked to consider included vegetation in streams, which could become exposed from inoculum carried by run-off following a heavy rain. As requested by Environment Canada we examined the health and status of the aquatic vegetation following exposure to spores of *C. purpureum* to look for possible non-target effects. These tests were important for determining recommendations for safe use of this biocontrol in riparian zones. Current guidelines for the application of chemical herbicides require the maintenance of a 10-m pesticide-free zone along all water bodies to prevent run-off contamination of the streams. Under natural conditions *C. purpureum* inhabits wounded deciduous trees at the edge of streams; hence it was expected that this requirement could be waived. We applied *C. purpureum* paste to stumps beside waterways and dispersing spores into the air column beside the water. From the results we concluded that there was no increase in spore levels from that already present. The material posed no risk to infection of the stream plant species. This provided one of the largest advantages of using this biocontrol as opposed to chemical herbicides as it may be deployed right up to the edge of streams and waterways. The only restriction imposed on it is that the product cannot be used within 50 m of fruit trees or ornamentals that may be pruned or grafted. This is not a concern as the material is intended for use in remote areas such as underneath power lines, pipeline rights-of-way and reforestation sites.

Efficacy and Use of *C. purpureum* as a Biocontrol

Development of the formulations is covered by de la Bastide (Chapter 32 this volume). Extensive field trials demonstrated that *C. purpureum* effectively prevents sprouting of cut stumps by colonizing and decaying the stumps (Fig. 31.2). The active ingredient, mycelium of *C. purpureum*, is formulated as a paste and is packaged in 1-litre squeeze bottles which contain a minimum of 1 kg of product having a minimum of 1×10^2 colony-forming units (cfu) per gram. One bottle is intended to treat approximately 200 cut stumps with diameters of 2–6 cm. Field efficacy trials were conducted on red alder (*A. rubra*), Sitka alder (*Alnus viridis* ssp. *sinuata*), speckled alder (*Alnus rugosa*), aspen (*Populus tremuloides*), white birch (*Betula papyrifera*), red maple (*Acer rubrum*) and big-leaf maple (*Acer macrophyllum*) by Mycologic Inc. in collaboration with BC Hydro Company, the Canadian Forest Service, the University of Victoria and Nova Scotia Agriculture College. The results of the field trials showed that on these deciduous trees the paste-formulated fungus had an efficacy comparable to that of chemical herbicides. *C. purpureum* was as effective as the herbicide Glyphosate™ in controlling red alder and was comparable to the chemical herbicide Release™ on Sitka alder. On aspen, the paste-formulated fungus showed statistically significant (compared to the control), but variable, efficacy of control, ranging from 36% in Ontario to 84% in British Columbia. On speckled alder and white birch, the



Fig. 31.2. With repeated cutting in a right-of-way a single alder stem had multiplied to form a clump of at least 12 stems. Cutting all of the stems followed by treatment with *C. purpureum* resulted in the complete suppression of re-sprouting from the cluster of stems. The fruiting structures were observed on the dead stems approximately 18 months following application.

biocontrol had a significantly higher efficacy (26% and 32%, respectively) than the control (0% and 1%, respectively). On red and big-leaf maples the biocontrol treatment was not effective (6% and 14%, respectively) as compared to the control (5% and 8%, respectively). For these reasons we are initially targeting areas on the west coast as the market, as here red and Sitka alders are especially problematic. As a follow-up to the joint registration of this biocontrol in both Canada and the USA we are exploring the use of *C. purpureum* to control invasive tree species in protected areas, especially in the southern areas of the USA.

Summary

The development of *C. purpureum* as a biocontrol was a long journey. However, the research plan we implemented led us to develop a product that very effectively and safely controls woody deciduous weeds at reforestation sites and other corridors where brush control is required. We are continually trying to improve the use of this product through new formulation and application technologies. For complete acceptance by the end-users a truly successful biocontrol product must be as effective and easy to use as chemical herbicides or pesticides. It is our expectation that every successful product developed from biocontrol research will help facilitate change and reduce our reliance on chemical interventions for weed or pest control.

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32

Developing the Production System for *Chondrostereum purpureum*

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Overview: This chapter details the commercial development of the phytopathogenic fungus *Chondrostereum purpureum* as a bioherbicide for controlling woody deciduous species in industrial rights-of-way and reforestation sites. Concerns associated with industrial production, regarding the scale-up of fermentation and quality control of the product, including consistency and efficacy of the product, contamination, shelf-life and formulation, are discussed. Each element of the production cycle has a cost factor and significantly affects the potential success of a product. The strategy for implementation of the technology into current management use also has major implications for successful product commercialization.

Introduction

The initial stages in the research and development of a biological control (biocontrol) product will very often, in hindsight, be regarded as the easiest part of the commercialization plan. Once the potential value of a biocontrol agent is identified under controlled environmental studies (e.g. the laboratory), the investigators will need to progress through a number of crucial milestones, which will present to them significant challenges, before the commercial viability of a control agent can be confirmed. These milestones include: (i) determining the range of target species that may be controlled; (ii) documenting product efficacy under realistic field conditions; (iii) establishing product safety for humans and other non-target species; (iv) integrating the new agent into existing strategies of target pest control; (v) developing a suitable product formulation; and (vi) designing a manufacturing process that will maintain product quality.

Our research group has been involved with the development of the phytopathogenic fungus *Chondrostereum purpureum* (Aphyllophorales, Corticiaceae) as a bioherbicide for the control of woody deciduous species colonizing industrial rights-of-way (ROW) and reforestation sites (see Hintz, Chapter 31 this volume). This fungus is a weak pathogen of a wide range of broadleaved tree species and is an early-stage colonizer of wounded trees. The colonization of fresh wounds

by basidiospores results in an infection of the sapwood with vascular decolorization and necrosis, resulting in stem canker formation. Pathogenicity of *C. purpureum* is expressed in some species as silver leaf disease, and mortality is usually only seen in trees that are severely stressed. A formulation of this bioherbicide was submitted for joint review by the Pest Management Regulatory Agency (PMRA) in Canada and the Environmental Protection Agency (EPA) in the USA, and a temporary product registration was granted; a full registration is pending and is expected to be approved in 2006 (PMRA, Regulatory Note REG2004-09). The following discussion will consider the development of a production system for *C. purpureum* as an example of commercialization of a microbial biocontrol organism.

The commercialization of any biocontrol agent requires submission of a detailed information package to support the registration of a new active ingredient. In order to identify all data requirements for product registration, it is of critical importance to consult at a preliminary stage of product development the regulatory guidelines and the agency responsible for the proposed jurisdiction of use. For *C. purpureum*, a pre-submission consultation with PMRA brought to light several areas of concern (e.g. the potential impact of dispersing basidiospores on non-target tree species in fruit tree orchards). It also identified data requirements for which waiver requests could be sought, with adequate supporting data from the relevant literature, such as the potential impact on non-target animal species in the environment. The identification of areas of concern prompted us to also focus on genetic marker development and studies of environmental fate for *C. purpureum*, so as to provide adequate data to support the safe use of this bioherbicide.

Scale-up and Product Quality

Scale-up of production remains a universal concern for biocontrol researchers, as it can greatly affect the efficacy of the manufactured biocontrol product. Large-scale fermentation often creates drastically different culture conditions compared with those found with laboratory-scale cultures. We were concerned that the fungus, when grown in a large-scale fermentation tank, may have lost its effectiveness for suppressing re-sprouting. We therefore carefully monitored the growing conditions during scale-up so that the desired traits were conserved. For this we measured variables such as morphology and phenotype of the vegetative mycelium and growth rate, and we confirmed the genetic stability with multi-locus genetic markers. These variables were first shown to be valid indicators for *C. purpureum* under lab-scale growth. Subsequently, they proved to be equally valid when large-scale fermentation was initiated (Boyetchko, 1999).

Large scale-up fermentation can affect shelf-life and thus the label guaranteeing the concentration of the technical-grade active ingredient (TGAI). The long-term viability (and shelf-life) of the fungus depends in large part on its physiological state at the time of harvest. The fermentation process influences the titre (colony-forming units (cfu)/g) of the TGAI and the quantity of material required to produce a standard formulated end-use product (EP). For any formulated EP,

a guaranteed minimum titre must be stated on the label as a measure of product quality and is usually linked to a time-frame (3, 6 or 12 months).

Fermentation Strategies and Shelf Life

Two fermentation methods are most often used for manufacturing microbial agents, namely liquid and solid fermentation. Liquid fermentation media are water based (80% or more water by weight), and are amended with required nutrients and substrates. Solid fermentation media have a much smaller proportion of water (30% or less) and may, or may not, be amended with nutrients. Either method of fermentation can be used to formulate the EP, depending on the particular requirements of the biocontrol agent. For production of *C. purpureum*, a two-stage fermentation process was utilized in order to combine the benefits of both liquid and solid processes. The first stage involved a stirred liquid fermentation in a defined medium under controlled conditions. This produced a high proportion of viable vegetative mycelium. This culture was then diluted with a volume of fresh liquid medium to obtain a pre-selected titre of the fungus. For the second stage, we used a solid substrate containing adequate moisture and a suitable nutrient supply. The diluted liquid fermentation was then added to the solid material to obtain the desired titre. The fungus colonized the solid substrate over several weeks of growth at room temperature, followed by transfer to 4–8°C for long-term storage. Using this process, the solid TGAI remained at an acceptable titre, ranging from 1×10^7 to 5×10^8 cfu/kg. The shelf-life of our product was found to depend on the format of the substrate used for long-term storage, and the solid substrate for the TGAI proved to maintain the highest titres of inoculum over the longest period.

Another important aspect of a fermentation strategy to be considered is the ease of storage and transport of the biocontrol product. The selection of a liquid versus a solid substrate for the TGAI and/or the EP influences both product storage and transportation requirements. A liquid fermented product, which is mostly water, is heavier than a solid material and thus more expensive to transport based on equivalent titres. If acceptable product quality can be maintained with a solid substrate and this format is compatible with the method by which the product is to be applied, then this may be the preferred substrate. The growth requirements of the microorganism, however, may be a more important factor in deciding which fermentation strategy is employed. In our case, using a solid substrate provided several benefits for *C. purpureum* bioherbicide production (Boyetchko *et al.*, 1998; Zidack and Quimby, 1998). The product could be stored until such time that the EP was needed. At that time, the quantity of TGAI required to obtain a label guarantee for a titre of 10^5 to 10^7 cfu/kg was removed and made into a paste that was to be applied manually to cut stumps. The solid substrate could also be transported to the location of use, where the EP formulation can be prepared; this has the advantage of both reducing transportation costs and maximizing shelf-life. By producing and storing large quantities of solid TGAI for future use in EP production, economies of scale were realized in terms of cost and time of production.

The latter element is highly useful for meeting seasonal or variable demand for a biocontrol product.

For some microbial biocontrol products, the formulation of the EP can be a bottleneck in the production process, for several reasons. Some formulations may require more care and attention, owing to the exacting requirements of the end-user at the site of use and the narrow tolerances of specialized application equipment, as well as a limited range of conditions under which the biocontrol agent will be effective. With our bioherbicide product, the advantages provided by producing a solid TGAI allowed us to formulate the EP in response to these limiting factors and improve overall production efficiency.

The demand for *C. purpureum* currently is for a liquid spray formulation that can be used in a backpack sprayer, modified brush saws and similar spray equipment. We are now testing EP formulation processes that provide a liquid suspension from the solid TGAI that is of sufficiently fine viscosity that it can pass through a small nozzle aperture without blocking the flow. The industry prefers sprayable formulations and we must respond to the need of the end-users.

Quality Control – Product Identity, Titre and Efficacy

The key elements to be considered for quality control of biological agents are product identity, efficacy and safety. A central component of our *C. purpureum* bioherbicide quality control programme was the ability to identify the active ingredient. Correct taxonomic identification, whether it is for an existing or an entirely new species, is an essential element for bringing a product to market. This includes morphological, physiological/biochemical and genetic characterization of the isolate. The ability to identify the fungus with certainty from a fermented pure culture, a host or soil, and to distinguish from a consortium of microorganisms is a requirement of product registration, for studies of product efficacy, environmental fate and the assessment of non-target impacts (including infectivity, pathogenicity and toxicity in a test subject). As already mentioned, monitoring species identity and genetic stability is essential during production. The identity of the *C. purpureum* isolate we used for our active ingredient was confirmed using a series of molecular markers. This species is well characterized, owing to its cosmopolitan distribution. As it is implicated in silver leaf disease of fruit cultivars, it was important to ensure good characterization of fungal genotypes to ascertain potential causality.

Quick and accurate methods for identification are of great help for maintaining product quality in large-scale production. Such techniques provide a means for rapid real-time quality control at the critical stages of production (e.g. master stock verification, start and end of fermentation runs, formulation production) and for monitoring product shelf-life. It also facilitates evaluation of product efficacy against new target species and the determination of environmental fate, as product acceptance often brings about new venues of use.

Our own research illustrates this approach. We developed genetic markers primarily to monitor the environmental fate of field-inoculated strains of *C. purpureum*, to evaluate strain efficacy in field trials, and to study the population structure of this species. These genetic markers were based on

SCAR (sequence characterized amplified region) PCR primers, which amplify a collection of variable-length fragments from the target genomic DNA of a *C. purpureum* strain. This method provided a unique, reproducible polymorphic banding pattern for each fungal genotype. It was also used to monitor strain stability. These sequences occur in multiple DNA fragments of variable length for a given *C. purpureum* isolate and are neutral in character, features well suited to their use as strain-specific genetic markers (Becker *et al.*, 2004). These markers are also useful in monitoring genotype identity and genetic stability during production, providing a reliable DNA-based method of quality control.

Quality Control – Product Purity and Safety

Protocols verifying product purity and safety were the other key elements of quality control in our production protocols for *C. purpureum*. Absence of contaminants must be established at critical stages of production (concurrent to active ingredient identification), specifically: (i) for the replication of Master Stocks; (ii) at the initiation of liquid fermentation; (iii) the inoculation of solid TGAI substrate; (iv) the formulation of the EP; and (v) the release of EP product for shipping. Tests for purity need to consider contamination by non-pathogenic microorganisms and known human/animal pathogens. Low levels of non-pathogenic contaminants (yeasts and moulds, aerobic bacteria/mesophiles) are permitted by some regulatory guidelines, but at no time are human and animal pathogens allowed to be present in the TGAI or EP. It is the responsibility of the manufacturer to ensure that appropriate quality control tests are included in the manufacturing process and that the regulatory authorities are notified in the event that a known pathogen is detected in a product. Standardized analytical methods for the detection of pathogens are available on public web pages and are updated as new analytical methods are described in the Laboratory Biosafety Guidelines (Public Health Agency of Canada, 2004) and in the Compendium of Analytical Methods (Health Protection Branch, 1999, 2002).

Cost Considerations for Growth Media and Inert Formulation Ingredients

An important aspect of production scale-up is the selection of suitable nutrient sources and substrate for growth of the microbial agent. Laboratory-scale experiments generally use high-purity laboratory-grade ingredients for growth media and growth substrates, in an effort to standardize conditions for the evaluation of potential biocontrol agents. For large-scale culture, less-refined, food-grade-quality ingredients offer costs savings and convenience of availability. The overriding provision, of course, is that they must provide an acceptable fermentation product with good viability, shelf-life, efficacy and safety.

Many inert ingredients (formulant ingredients other than the active ingredient) have been used to produce registered pesticide products in Canada and the USA, and lists of these are available at net sites of the PMRA and the USA EPA (PMRA, List of Formulants REG2005- 01; US EPA Inerts List). Both organizations make distinctions between those inerts that are of toxicological concern (List 1), potentially toxic (List 2), inerts of unknown toxicity (List 3), inerts of minimal risk (List 4A) and those for which the current use pattern will not adversely affect public health or the environment (List 4B). It is important to consult these lists before a product formulation is adopted for testing of a biocontrol agent, in order to identify potentially useful inert ingredients, with a history of use in pesticide products, and to confirm the safety of selected ingredients. The selection of more widely used and recognized as safe ingredients may offer a cost advantage and enhance the saleability of a biocontrol product.

Integration into Existing Application Methods of End-users

Where and how a biocontrol agent is ultimately used is an important consideration in designing a production system. The use of any biocontrol agent, whether it be in a greenhouse, agricultural field, forest site or an urban setting, must be integrated into the existing methods and strategies of pest control in that setting. A successful biocontrol agent will probably be integrated into a pest management strategy (Charudattan, 2000, 2001). For *C. purpureum*, we knew that the fungus had to be applied to tree stumps within a short time after they were cut, in order to maximize the susceptibility of the exposed tissues to infection. This would also reduce the effects of environmental constraints potentially imposed on the fungus that could limit successful colonization of the cambial tissue (e.g. max/min air temperature and occurrence/amount of precipitation). Trees in a right-of-way or a forestry site are usually cut with a chainsaw or brush saw, and sometimes by mowing. The cutting operation cannot be impeded by the method used for application of the biocontrol organism, nor should it require much additional labour. Our initial EP formulation was a paste that was applied manually to the cut stump. However, this method of treatment is labour-intensive and we are now testing a new sprayable formulation, which will reduce application time. We do not believe that this will add any additional cost to the product, but it will increase the product acceptance. The associated cost of labour in the use of this bioherbicide, in addition to product efficacy, is a major consideration for end-users.

Consultation with the expected user groups and their professional associations can help to identify where needs exist for new biocontrol agents. Determining the likely conditions of use and the most appropriate method of product application helped to guide our formulation development and contributed to a compatible manufacturing process to meet the objectives of the end-user. We have promoted our product through seminars and technical notes in order to introduce the idea of this new pest control strategy to potential future user groups. Having a history of knowledge available for a product enhances its adoption, not only by customers but also by decision makers.

The Cost of Biocontrol Products to the End-user

The successful marketing of a biocontrol product requires that it be cost competitive with existing methods of pest control and that it has a similar level of efficacy. This was our objective in the development of the *C. purpureum* bioherbicide product. However, the inherent nature of most biocontrol products is that they serve a niche market for a relatively small user group. Consequently, they are often more expensive in terms of their development costs, and the relatively small-scale production volumes need to be recovered over this small market size. Competing with conventional pesticides with their broader host range and longer history of use is challenging. A biocontrol product, however, can be successful in a niche market where pesticides are ineffective due to target resistance, or where pesticide use is restricted by legislation (Holt, 1992; Beckie *et al.*, 1999; see Boyetchko *et al.*, Chapter 30 this volume). For both the right-of-way and forestry sectors, there is increasing pressure to move away from conventional chemical herbicide use; few alternatives exist for vegetation control, other than the brushing of woody vegetation. In this situation, *C. purpureum* bioherbicide is seen as a viable alternative, if we can make it cost competitive with brushing. Biocontrol strategies have made significant incursions into use within the horticultural and agricultural sectors, where the harvested product can be marketed as pesticide free (Jarvis, 1995; Willer and Yussefi, 2004). For pesticide use in conventional markets, the development of integrated pest management strategies has placed a higher value on biological methods of control as part of a balanced approach to pest management (Jarvis, 1995; Glare and O'Callaghan, 2000).

Cost competitiveness can be significantly increased by large-scale production of the biocontrol product. In some cases, partnering with established manufacturing companies with existing infrastructure for microbial production and a distribution network can offer significant advantages to small companies. By considering both Canadian and foreign sales, limited niche markets can be expanded. International sales can be assisted by selection of suitable partners for manufacturing. The *C. purpureum* bioherbicide was developed for both Canadian and US markets, with vegetation managers in the utility and forestry sectors being the targeted market.

Summary

The development of a production system for a new biocontrol product involves looking forward many steps in product development. It is vital to understand and comply with regulatory requirements for any new product. A thorough understanding of the target market and of the user group is essential for the determination of optimal formulations, application methods and use strategies. Cost considerations will always be a primary component for all aspects of product development and must be part of the equation when evaluating commercial viability.

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33 *Beauveria bassiana* for Pine Caterpillar Management in the People's Republic of China

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Overview: Pine caterpillars are common forest pests in China, causing severe defoliation and economic loss. This story describes the 36-year history of a successful biological control programme using *Beauveria bassiana* for pine caterpillar management in China and the gradual development of an unusual but effective application strategy.

The Pine Caterpillar Problem Is Not Resolved Through Use of Chemical Insecticides

Pine caterpillars, *Dendrolimus* spp. (Fig. 33.1), are common forest defoliators from north-eastern to southern China. Twenty-seven species in this genus are found, and among them, the Masson's pine caterpillar, *Dendrolimus punctatus*, is the most common in the south, ranging from the Huaihe River southwards to Vietnam. Generally the insect defoliates 2-year-old pine needles, and outbreaks occur about every 3 to 5 years. On average, losses are about 1.425 m³ growing stock of the Masson's pine stands. During outbreaks, severe defoliation occurs with only the newly emerged needles remaining, and when such defoliation occurs repetitively, the trees usually die.

From the 1950s, the primary means of control was applications of wide-spectrum organochlorides such as DDT and benzene hexachloride (BHC). In the 1960s, these products were replaced by organophosphorous insecticides such as dipterex and DDVP, primarily governed by the strategy to 'treat early and in an eradicated manner while the pest is young'. Such misuse of chemicals caused serious problems, which started in the late 1960s when outbreaks became more and more frequent, even yearly, with population densities reaching more than 1000 larvae per tree. This was far in excess of what is considered as the threshold for treatment of five animals per tree. The caterpillar migrated over pine stands, crop fields and roads in search of intact pine trees, and even invaded houses. They even crawled up on human bodies and sometimes caused serious



Fig. 33.1. Masson's pine caterpillar, *Dendrolimus punctatus*.

skin disorders known as *Dendrolimus* osteoarthritis. Concurrently, resistance to insecticide rose rapidly, resulting in more frequent spray applications. With this came reports of poisoning of some pesticide applicators.

History of Mass Production and Use of *Beauveria bassiana* Against Pine Caterpillars: the Early Years

The first trial of the use of *B. bassiana* in China was against the sweet potato weevil, *Cylas formicarius*, by Boxing Lin (1956) of Fujian Agricultural College in Fujian, south-east China. Shortly after, in 1958, Yunwei Li of the Forestry Institute of Fujian Province conducted the first trial with *B. bassiana* against the Masson's pine caterpillar (Fig. 33.2). Nearly 500 kg of dried culture was produced in jars and applied as a mist spray on nearly 100 ha of Masson's pine, *Pinus massoniana*, plantations, the most important pioneer tree for reforestation in southern China. The trial resulted in an average mortality of 82% of the insects on the 8th day, compared with only 10% in the control. In addition to production of the *B. bassiana* conidia using solid substrate, they also conducted trials for production in submerged fermentation. They found a serious problem with this method, however, as the blastospores produced in liquid culture were short lived and therefore of no practical value. In 1969, new studies were started in Xinhui County, Guangdong Province, where a mass-production system was developed on inexpensive and easily obtainable grain by-products. As a result, nearly 2000 ha of Masson's pine were treated by spraying *B. bassiana* as conidial mists and dust. They also released live larvae that were pre-treated with the fungus, to increase the fungal epizootic within the insect population (Li *et al.*, 1981). Satisfactory results were obtained and this project was extended by the central government.

In the spring of 1970, the Ministry of Agriculture and Forestry held a workshop for 13 southern provinces. At this meeting, the low-technology mass production and large-scale application for pine caterpillar control were encouraged. Within a short time hundreds of small plants producing *B. bassiana* appeared throughout China. This fungus, as well as other species of fungi, was tested against dozens of forest and crop pests, making 1970 a milestone in the Chinese history of entomopathogenic fungal application and microbial pest control.

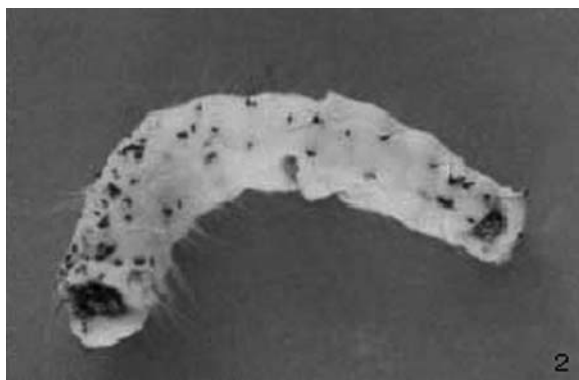


Fig. 33.2. A cadaver of the Masson's pine caterpillar infected by *B. bassiana*.

During the 1970s, the biocontrol programme using entomogenous fungi was supported by both central government and local governments. Although the Masson's pine caterpillar was the most important pest, targets were extended to other pine caterpillars, including the Chinese pine caterpillar, *Dendrolimus tabulaeformis*, in northern China, the Simao pine caterpillar, *Dendrolimus kikuchii*, in southern China, and even the larch caterpillar, *Dendrolimus superans* (= *Dendrolimus sibiricus*), in north-east China. Thousands of metric tons of the products were mass produced using low-cost technology. Hundreds of thousands of hectares of pine plantations were treated, but no accurate figures of production were kept.

The low-technology production of *B. bassiana* was mainly by solid-substrate culture in various kinds of containers with large surface areas, such as wide-mouth jars (mostly glass jars) or pots, or on various kinds of bamboo or wooden shallow trays (Fig. 33.3) or mats. Various grain products were used as media, mainly wheat bran, sometimes mixed with rice bran or corn meal. The products were mostly non-formulated dried culture or simple formulated powders with some fillers added, mostly gaolinite or yellow subsoil.

In the 1980s, however, the situation changed gradually, partly due to less direct government financial support and partly due to importation of cheap chemical pesticides under the government's new policy of *Reform and Opening-up*. Most *Beauveria* plants closed and fewer than 50 plants survived with various kinds of government support. Since then, the remaining plants have attempted to improve their labour-intensive technology for mass production. Various techniques of biophasic fermentation and semi-solid fermentation were developed to increase yield and conidial content, decrease contamination and enhance mechanization. However, few such attempts resulted in novel means of large-scale industrial production. To date, almost every plant is small and not well organized as a commercial operation. Their products are not registered and most of them are not formulated. They have been producing the fungus mainly for local use with provincial government subsidies to the *Beauveria* users. As well, the National Bureau of Forestry directly subsidized a few *Beauveria* plants at the beginning of the new century. However, the production and application of *B. bassiana* has been drifting away from *The Regulation of Pesticides of P. R. China*, issued by the State Council on 8 May 1997. The current status is that *B. bassiana* is mass produced by low



Fig. 33.3. Shallow tray culture of *B. bassiana* in a small plant in the 1970s and 1980s.

technology at a small scale in many small plants and used on a large scale as one of the main measures against pine caterpillars throughout southern China.

Improvements in the Mass Production of the Fungus

I had been conducting research on improving techniques of mass production for local use as a forest engineer and head of a *Beauveria* plant in a state-owned forest farm from 1971 until 1979. In 1975, I wrote and published a booklet consisting of 90 pages and 40 illustrations entitled *Mass Production and Use of Beauveria bassiana*. It was regarded as an introduction to the fungal insecticide programme and a manual for workers in the field of forest pest control for more than a decade. In 1979 I started my career as a faculty member in the Anhui Agricultural University. I went to the US Department of Agriculture, Agricultural Research Service, Insect Pathology Research Unit at Cornell University, to learn new technologies and study new entomopathogenic fungi, mainly entomophthoralean fungi. Sadly when I returned to China in 1983, I found that the mass-production techniques for *Beauveria* had made no progress. The only great achievement was the development of equipment for extraction of the conidia. Its wide adoption in the mid-1980s helped every *Beauveria* plant get rid of allergies caused by inhalation of conidia during milling and packing the conidial powder.

During the second half of the 1980s, the Ministry of Agriculture, sponsored by Qingfeng Xu, funded a project for improvement of mass-production techniques. In the course of this research, they made great progress in the techniques of solid-state fermentation using porous materials such as rice shells or pieces of cork inoculated with thick submerged culture, mycelia and blastospores. High concentrations of conidia were obtained on such porous substrates, but the techniques were not stable and therefore were abandoned. Meanwhile, safety of *B. bassiana* to humans was systematically tested by Qingfeng Xu and his research group in collaboration with China Medical University. Testing covered acute and chronic toxicity, pathogenicity, allergy, carcinogenicity, teratogenicity and mutagenicity. All results indicated safety to humans. However, no one was concerned

about the registration of the fungal insecticides because laws concerning the regulation of pesticides were not issued until 1997. In most cases, the low-technology mass production was proceeding as usual, suggesting that the technological improvement and commercialization of fungal insecticides in China was a long way away.

My Return to *B. bassiana*

After a 12-year break from the project, working on Chinese resources of entomophthoralean fungi from 1983 to 1991, I returned to the field of using *B. bassiana* against forest pests. I organized a team of scientists to work on improvement of isolates, mass-production techniques, formulation and forest application techniques from early 1991 through late 1995. During these 5 years, we found that saltation occurred frequently in subculturings on artificial media due to heterokaryosis, heteroplasmosis, and/or even DNA recombination (Tang *et al.*, 1996), causing strain degeneration within 5–14 subculturings, decreased sporulation and virulence. To decrease the saltation and the degeneration, we found that fermentation conditions such as medium, temperature, humidity and light had to be manipulated. A medium made of wheat bran, comparatively low temperature (22–25°C), low to medium moisture and proper diffuse light caused the least saltation.

We improved techniques for biphasic fermentation to enhance mechanical utilization and production stability. We developed oil and powder formulations, which were more stable and easier to use. There were no available commercial production techniques for large factories equipped with big industrial fermentors. Therefore, in 1996, I chose to work with the Zhongbang Bioengineering Company of Anhui located in Hefei, which was equipped with two 25 m³ fermentors designed specifically for solid culture of *Bacillus thuringiensis* by the Institute of Process Engineering, Academia Sinica. It took us 3 years to train workers and to adapt the technology for surface culture of a fungus. Although we were able to produce large quantities of fungus, the project ended due to financial difficulties that the company encountered.

From the beginning of the new century, I started to look for a new partner for commercialization of *B. bassiana*. The company Tianren Group located in Ji-an, Jiangxi was selected as it was equipped with eight solid fermentors of the same type as those of Zhongbang (Fig. 33.4). I was surprised that they had already received many orders for tons of *Beauveria*, targeting various important pests including coconut chafers, greenhouse aphids and whiteflies, the peach fruit moth, *Carposina nipponensis*, and the lesser green leafhopper, *Empoasca flavescens*, in addition to the main forest pests, Masson's pine caterpillar and the pine sawyer, *Monochamus alternatus*. This indicated that there was a large market for such products, certainly well beyond what they expected and beyond their production capacity. I promised to supply them with proper isolates from our entomopathogenic fungal culture collection of more than 1700 isolates at the Anhui Agricultural University. While they improved their equipment and techniques to decrease their intolerable energy consumption, 2 years of field efficacy tests were carried out as part of the requirements for registration by the Ministry of



Fig. 33.4. Solid fermentors in a company in the early 2000s.

Agriculture, in hope that the first registered product would soon be made available. By July 2006, five products, based on pure conidia and non-woven fabric bands of *B. bassiana* and *Metarhizium anisopliae*, and an oil suspension, were finally registered.

Methods and Strategies of Application for Control of Masson's Pine Caterpillar: Inoculation versus Inundation

From the early 1970s through to the mid-1980s, raw products, i.e. dried cultures, either milled or non-milled, were applied through regular manual or mechanical sprayers. These applications were very labour intensive, especially in pine plantations on rugged and water-deficient hills. In contrast, application of dusts was more economical and was widely used for large area applications. Either processed (milled, primitively formulated or unformulated) or unprocessed powders could be spread by manual or mechanical dust sprayers (Fig. 33.5) and carried long distances by the wind. Field efficacy was estimated by caterpillar mortality and varied greatly from none to more than 90%. During the course of applications against pine caterpillars, some innovative and unusual application methods were developed to save on labour and treatment costs. These include:

- 1. Conidium fireworks.** A firecracker was wrapped with conidial powder (Fig. 33.6), and thrown up to the crown, where it exploded, sending a dust of conidia over the canopy. For high-altitude release, a mortar was used to launch the firecracker high into the air (Fig. 33.6). Recently, more sophisticated mortars were developed specifically for launching the firecrackers of conidial powder (Fig. 33.7), which resulted in much easier and more effective distribution of *B. bassiana* conidia.
- 2. Use of explosives.** Explosives were used at some forest farms to release conidial powder. Black powder was placed in the bottom of a big pit and then conidial powder was poured over the explosive. The explosion spread the conidia over a wide area (Fig. 33.8). Owing to the dangers involved in using explosives, this method was discontinued in the mid-1980s. Furthermore, this method required large

amounts of conidial powder and there was the possibility of damage to the conidia, but this was never evaluated.

3. Release of inoculated healthy caterpillars. This method was named metaphorically ‘tiger release into the mountain’. A worker walks in a pine plantation carrying the conidial powder or conidial suspension, collects some healthy pine



Fig. 33.5. Mist spray in the 1970s.

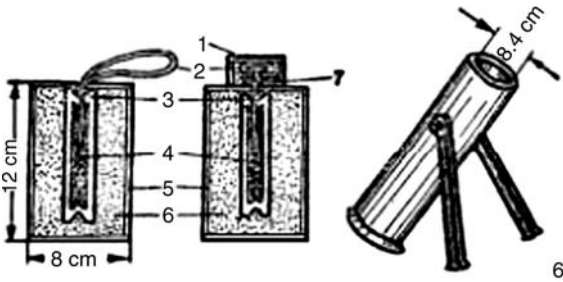


Fig. 33.6. Firework mortars of *B. bassiana* conidial powder in 1975 (Li, 1976).



Fig. 33.7. A newly developed mortar for launching the fireworks of *B. bassiana* (Downloaded from <http://www.cqwb.com.cn>).



Fig. 33.8. Widespread application of *B. bassiana* by explosion in the 1970s.

caterpillars, dips them in the powder or the suspension and then releases the inoculated caterpillars.

All of these novel methods were aimed at inoculative application, attempting to cause local infection and epicentres of mycosis: small epicentres by the firework and released caterpillar methods and larger epicentres by the explosives method. Owing to inaccessibility of the rugged and thorny forests, it was difficult to inundatively treat the forests using ground equipment. However, I found that populations of the caterpillars of the whole area declined dramatically sooner or later, regardless of the application method used (e.g. inundative or inoculative). After conidial application, the low population status persisted for at least 3–5 years, suggesting strong horizontal and vertical dispersal ability of *B. bassiana*. I proposed that the use of *B. bassiana* should be inoculative applications of small quantities of conidia to achieve prolonged suppression of pine caterpillar populations in as large an area as possible (Li, 1978). More proof was needed, however, to support this recommendation.

New funding provided me with an excellent opportunity to further study and compare the efficacy between inundative and inoculative releases in forests in southern Anhui between 1991 and 1995. I set up an experiment in three close forest farms of similar form, climate and vegetation structure. Each farm was treated using a different application strategy with *B. bassiana*. The results showed that the caterpillar populations on the three farms were all at low levels in 4 years, but the population dynamics were completely different. At the Magushan Farm (Han *et al.*, 1996), the pine stands were treated inundatively with *B. bassiana*, and the average population density was 3.2 ± 4.9 larvae per tree, with sharp fluctuations and sometimes with population levels close to or above the control threshold of five per tree and the highest population at 16.4 per tree. At the Jinsishan Farm (Han and Li, 1997), small area epicentres of caterpillars were accurately detected and treated with chemicals primarily and *B. bassiana* alternatively, with the average density at 1.5 ± 2.3 and highest density at 8.3 larvae per tree, which is above the economic threshold. At the Daigongshan Farm (Liu and Han, 1995), however, inoculative application of *B. bassiana* was used for 20 years, and the population levels remained low, with an average density at

0.1 \pm 0.1, and never above 0.6, providing us with a wonderful example of sustainable control of the caterpillars by inoculative releases of *B. bassiana*. Even in Magushan, the caterpillar population has been at low levels since they adopted the strategy of inoculative release after 1995.

In order to understand the process and mechanism of such sustainable control, I investigated biodiversity at the three farms. The results revealed that the species diversity at Daigongshan, where inoculative applications of *Beauveria* were used, was greater and more complex compared with Magushan and Jinsishan, where inundative *Beauveria* applications and *Beauveria* combined with chemical insecticide applications were used (Li *et al.*, 1998). This increased species diversity should contribute to community stability.

Further experiments to optimize the dose and frequency of the inoculative release strategy were made using 12 combinations of four doses (37.5, 75, 150 and 300 g of pure conidia per ha) and three frequencies (once and twice a year and once every 2 years). The results indicated that one inoculative release per year with 37.5 g conidia per ha resulted in the highest biodiversity index and lowest caterpillar population, suggesting an optimal inoculative release rate. I was also curious to find out the fate of the *B. bassiana* released by us during the sustainable suppression of the caterpillars with low-level inoculative releases. I found 127 different isolates of *B. bassiana* in just 1 year from 30 different insects as well as a small portion from soil, litter and air, revealing that there were many indigenous strains of *B. bassiana* and that they displayed great genetic diversity. I also found isolates of six other entomopathogenic fungi, but *B. bassiana* was definitely the dominant species.

These *B. bassiana* isolates could be attributed to 32 types from esterase isozyme profiles. Based on RAPD profiles they were attributed into seven RAPD genotypes. Each type could represent at least a branch in the food web, and the types connected by some hosts, making the food web very complicated. While I found the locations of the released *B. bassiana* during low population levels of pine caterpillars and how the population level of *B. bassiana* changed over the course of a year (Wang *et al.*, 2002), I was also convinced that both species diversity and genetic diversity played important roles in such sustainable suppression.

Based on these data, I can now clearly hypothesize the mechanism of the sustainable control of the Masson's pine caterpillar: protection of predators and parasitoids owing to reduced use of chemicals, persisting population of the released *B. bassiana* in soil, and especially host transfer. When the target host is at a low level, the artificially released strain can find one or more proper hosts on which to survive from abundant hosts through host transfer; when the released strain is at low level, the *B. bassiana* population of abundant indigenous strains can provide proper strains to suppress the pine caterpillar, also through host transfer. Host transfer connects species diversity and genetic diversity (Ding *et al.*, 2004). Furthermore, I propose that the long-term application strategy of inundative release of fungal insecticides should be substituted by a strategy of inoculative release for forest pest control. While I have been claiming such an unusual proposition at various national and international meetings, inoculative application is still being practised at a large scale against forest pests in China, either consciously or unconsciously, regardless of the users knowledge of my

proposition and whether or not they support it. However, their inoculative release needs to be optimized to be more ecologically sound, economically worthwhile and feasible.

Summary

To sum up, the story of use of *B. bassiana* against pine caterpillars in China is one of the most successful biological control programmes in the world. Technically, it is immature compared with the highly commercialized *B. thuringiensis* production, but it is mature from a practical point of view, especially for its important position in Masson's pine caterpillar control, economic benefit and public acceptance. Generally speaking, it is the story of a slow but successful development of a biological control programme. Progress should continue possibly for 5–10 more years while substantial improvements of commercial production are made. Application of *B. bassiana* against pine caterpillars will be further enhanced to meet the requirements of the National Bureau of Forestry, the former Ministry of Forestry, in that biological control should account for 60% of the general treated forest area by the turn of the century.

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34 Green Muscle™, a Fungal Biopesticide for Control of Grasshoppers and Locusts in Africa

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Overview: Locusts and grasshoppers are some of the most devastating insect pests known to mankind. This is the story of the formation of LUBILOSA, a consortium of agencies funded by numerous countries, whose mission was to develop a microbial control agent to fight these notorious pests. After 12 years and expenditures of US\$15 million, a registered biopesticide is born.

The Insects

The desert locust, *Schistocerca gregaria*, is one of the most notorious insect pests. When populations build up, locusts exhibit gregarious and migratory behaviour, leading to the formation of spectacular swarms with an enormous potential for crop destruction. The potential invasion area of desert locust swarms covers 20% of the earth's land surface, reaching from the West African coast to the Indian subcontinent. From mention in biblical texts to recent media reports, locust plagues attract public attention in a way that no other insects do. They feed on almost anything that is green and of plant origin. Crop losses caused by locusts are reported to be without comparison, but surprisingly only few reliable crop loss estimates are available in the literature. Since the last major outbreak of the desert locust in the Sahel between 1986 and 1989, most years passed with no upsurges, or only small upsurges in some years. However, at the beginning of 2004, the desert locust started causing havoc once more, impacting a part of the world that is for many different reasons ill prepared for handling such an immense, complex problem. The total area treated with insecticides since the beginning of the upsurge in October 2003, has exceeded 12 million ha, according to the United Nations Food and Agriculture Organisation's (FAO) Desert Locust bulletin. Owing to weather conditions and successful control operations, the situation calmed down again until the end of 2005.

The desert locust may be the most spectacular of the Sahelian pests but it is only one out of several acridids of economic importance. Less migratory and less gregarious acridids are called grasshoppers. Although grasshopper outbreaks might be less spectacular than those of the desert locust, the accumulated damage of grasshopper attacks is much higher on a per annum basis (Duranton *et al.*, 1981).

In West Africa, most spray campaigns are aimed at the Senegalese grasshopper (*Oedaleus senegalensis*). This species breeds in the rainy season throughout the Sahel, and often forms swarms which attack ripening millet.

LUBILOSA is Born

During the 1986–1989 locust outbreak, almost US\$300 million were spent on the application of 15 million litres of chemical pesticides to control grasshoppers and locusts. The resulting concern about environmental and toxicological issues stimulated studies on the development of microbial insecticides based on Deuteromycete fungal spores for the control of locusts and grasshoppers (Bateman *et al.*, 1993a; Johnson and Goettel, 1993). Over the last 15 years, research on alternative locust and grasshopper control methods has focused on entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* as potential commercial microbial control products.

In Africa, the LUBILOSA (Lutte Biologique contre les Locustes et les Sauteriaux) programme was established in 1989 and developed an oil-based formulation containing the fungal pathogen *M. anisopliae*. At the beginning, the project involved scientists from CABI in the UK, the International Institute of Tropical Agriculture (IITA) in Benin, and the Département de Formation en Protection des Végétaux (DFPV) in Niger, including us, the authors of this chapter. By the end of Phase 3 of the project, the number of collaborators had increased to include Comité permanent Inter-état de Lutte contre la Sécheresse au Sahel (CILSS), Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) in Germany and a private company, Biological Control Products SA (BCP), in South Africa. Funding came from the governments of Canada, Switzerland, the Netherlands, the USA and the UK. Overall, the LUBILOSA project has spent more than US\$15 million over 12 years.

The Search for a Candidate Control Agent

LUBILOSA, in collaboration with national plant protection agencies, screened grasshopper and locust populations across Africa to find indigenous Deuteromycotina fungi that attacked acridids. Our objective was to find a strain that was not only indigenous to the area but also efficacious against locusts and grasshoppers, was amenable to application through conventional spray equipment, could be economically mass produced, was able to survive the high temperatures characteristic of the Sahel, was stable when stored and was safe to humans, non-target organisms and the environment. *Metarhizium* was more common in Africa, while other

research established that *Beauveria* was more common in North America and the Mediterranean.

We accumulated more than 180 isolates of *Metarhizium* and *Beauveria* spp., 50 of which were *Metarhizium* strains virulent to Orthoptera. Although at first we identified these isolates as *Metarhizium flavoviride*, colleagues later established that these strains belong to a distinctive variety, *M. anisopliae* var. *acridum* (Driver *et al.*, 2000), which attacked only grasshoppers and locusts. One of our isolates, strain IMI330189, was selected as type material for this variety. This isolate was initially found by a colleague scientist in Niger on a cadaver of a dead grasshopper (*Ornithacris cavroisi*) outside of the ICRISAT (International Crops Research Station for the Semi Arid Tropics) station. This isolate was well adapted for Sahelian conditions. It was finally selected for the development of a product for locust and grasshopper control, which came to be known as Green Muscle™.

Mass Production

One of the greatest challenges of wide-area use of microbial control is to find an economical means for mass production. The LUBILOSА programme established a pilot plant for the production of Green Muscle™ at IITA in Benin. This plant enabled us to produce sufficient high-quality spores to carry out large-scale field experiments using aerial application, which is very common in grasshopper and locust control. Fungal spores are produced in a simple two-stage system: in liquid yeast-sugar broth and on sterile rice. This process was successful because we implemented a rigid quality control. Strain IMI 330189 competes poorly with contaminants, requiring careful control of production parameters, which is only possible in specially built facilities (Jenkins *et al.*, 1998). Analyses showed that an intermediate technology production unit, like the one at IITA, with a production cost of US\$200 per kg of spores, was not commercially viable. Consequently, Green Muscle™ is currently produced using a more sophisticated process. The industrial process is highly automated and spores are produced in large solid-substrate fermenters. Production parameters can be controlled more easily. It requires much less manpower, replacing labour costs with capital costs. However, the LUBILOSА production unit still serves as an excellent model for a non-commercial, medium-scale mycopesticide production, which can be attractive for different pests under different economic conditions.

Field Performance

The host range of *M. anisopliae* var. *acridum* is narrow, and at field application rates, it is safe to most non-target organisms (Langewald *et al.*, 2003), making it an environmentally sound product. The lack of toxicity of *M. anisopliae* to mammals, and consequently to humans, offers a further advantage over old technology insecticides such as organophosphates.

In the field, Green Muscle™ is applied in the same way as a chemical pesticide. IMI 330189 dry spores are formulated in oil and applied at very low

application rates of 25–75 g per hectare ($1.25\text{--}3.75 \times 10^{12}$ spores per hectare) and at a volume of 0.5 to 2 litres. Ultra-low-volume spray application is an excellent way to release a relatively high concentration inoculum of the fungus into the host organism's environment. The inoculum reaches the host insect's cuticle either directly through spray droplets or indirectly through the spores released by the spray residue on the vegetation or soil. The fungus kills its host by germinating on the host insect's cuticle, penetrating it and growing inside the host's body. This process takes time, and speed of kill is slow when compared with synthetic insecticides. This is often considered as a major disadvantage of the product. However, we know it is not necessarily death which makes insects stop causing damage.

Some other parameters associated with the use of the biopesticide are extremely important in the overall efficacy of Green Muscle™. We demonstrated that infected grasshoppers consume less food (Thomas *et al.*, 1997) and are more prone to predation than healthy individuals (Thomas *et al.*, 1998). Depending on meteorological conditions, after death cadavers sporulate, producing new fungal inoculum, and in addition the spray residue continues to infect grasshoppers over a long period. The long persistence of fungal spores in the field, which under optimal conditions can accrue to more than a year, plays a very important role in the overall efficacy (Langewald *et al.*, 1999). The activity profile of Green Muscle™ looks different when compared with a knock-down insecticide. In large plots treated with Green Muscle™, we achieved a reduction of Senegalese grasshopper by more than 95% 16 days after application, and it remained low until 3 weeks or more after application, while in organophosphate-treated plots, fast knock-down was achieved but with only a short residual effect, and grasshopper populations climbed back to initial population densities over the observation period (Langewald *et al.*, 1999).

Biological Constraints

Together with our colleagues of the national programmes, we successfully tested Green Muscle™ against many locust and grasshopper species (Lomer *et al.*, 2001). To demonstrate the high efficacy of Green Muscle™, given a relatively slow speed of kill and the extremely high mobility of the target organisms, required large field trials with plot sizes of up to 1000 ha. Because of the rare likelihood of outbreaks, the high mobility of the target and the remoteness of field sites, we found demonstrating efficacy in desert locust particularly difficult, and sufficient data from large-scale field experiments are few. In initial experiments, the programme demonstrated a reduction in size of treated hopper bands compared with non-treated controls (Langewald *et al.*, 1997) and during a recent large field trial in Algeria, all hopper bands treated with Green Muscle™ became sick and were subsequently eliminated by birds and lizards.

Apart from slow speed of kill, another constraint to the use of Green Muscle™ that we found was the capacity of many grasshopper species to regulate their body temperature by exposing themselves to solar radiation (Blanford *et al.*, 1998). This behavioural feature slows down the growth of the fungus and might, under certain circumstances, suppress fungal infection completely. Under very

hot environmental conditions, which allow grasshoppers to slow down fungal growth, or under cold weather conditions, which reduce the growth rate of the fungus as well, the overall efficacy of Green Muscle™ might be very slow but still satisfactory within one generation cycle. However, we anticipate that it may be difficult to market a product when feeding reduction or dead insects are not immediately visible, which leads us to the economic environment of grasshopper and locust control in Africa.

Economic Constraints

Green Muscle™ was developed for a fragmented, difficult and very low-value market. The Sahel represents one of the most difficult agricultural environments on Earth. In this environment, grasshopper control has always been difficult to justify in purely economic terms. Generally, for food crops, farmers are not ready to invest in any inputs, and in case of emergencies due to grasshopper or locust outbreaks, provision of food aid may be cheaper than mobilizing expensive locust control operations. The current price of Green Muscle™ works out at US\$20/ha for a dose of 50 g/ha, which is 1.5–2 times the price of conventional chemical insecticides, but there is scope for reduction of application rates. However, cost–benefit calculations are difficult to apply in subsistence farming systems, where only a minor proportion of the crop reaches the commercial marketplace.

Apart from logistic and socio-economic problems, such as disruption of social structures, distortion of local food markets and unemployment linked to food aid, there are other externalities connected with grasshopper damage that justify control operations, which are difficult to estimate but warrant evaluation. Additionally, in West Africa, distributors are almost completely absent from the grasshopper and locust control market. Grasshopper and locust control is therefore almost completely dependent on national plant protection organizations and international donor agencies. Because of ever-changing political priorities, multinational intervention schemes rarely provide sufficient long-term stability for effectively managing sporadic pests such as locusts and grasshoppers.

Large agrochemical companies consider this market as too small and unreliable to justify the costs associated with developing specific products for the locust control market. So far the cost for developing Green Muscle™ has accrued to more than US\$15 million, and donor agencies that are committed to development aid usually find it outside of their mandate to provide funds for what essentially amounts to a commercial enterprise.

The development of any commercial product for plant protection has strong regulatory implications, and the registration of microbial pesticides requires toxicological and ecotoxicological studies. The number of required tests is less compared with chemical pesticides but, given the expected low market share, proportionally at least as expensive. Some of the efforts for registration of Green Muscle™ were covered by our programme, and most environmental data were generated by ourselves, or partner organizations. Meanwhile Green Muscle™ is registered in the CILSS countries (West African Sahel), South Africa, Namibia, Zambia, Tanzania and Sudan. However, in order to cover a sufficient market in

Africa, Green Muscle™ will need to be registered by many additional countries or regional organizations.

Until now, Green Muscle™ has failed to gain a significant market share. The total area treated has not exceeded 20,000 ha, equivalent to approximately 1.5 t of spores, worth US\$450,000 in production costs. To a large extent, the product was purchased by agencies such as FAO or the Luxembourg development agency mainly for field testing. In the Sahel, the plant protection agency of Niger was for many years the most active organization in grasshopper control, carrying out large aerial control operations. The agency was supported by the government of Luxembourg, with a great interest for biological grasshopper control. In 2000, Green Muscle™ was officially launched in Niger, and the prospects for selling large quantities were excellent, because of its proven efficacy on Sahelian grasshoppers. When an order over US\$100,000 was pledged, the producers of Green Muscle™ were not able to deliver. In 2002, the government of Luxembourg changed priorities in aid policies, and an important source of funding for biological pesticides disappeared. FAO continues to show interest in the use of Green Muscle™ for desert locust and red locust control, though not all large field trials sponsored by them were conclusive. The current desert locust outbreak situation is a new opportunity to demonstrate the efficacy of Green Muscle™ on desert locust in large field trials. Recent results from Algeria show that the product controls hopper bands. Interestingly we observed again that most hoppers were eaten by predators before they died from the fungal infection.

Even though experts are calling for microbial control solutions to be viewed with a different mindset from conventional pesticides, the reality at the end-user level is that performance (speed of kill and consistency of control) and price will continue to be benchmarked against conventional pesticides.

Giving Microbial Grasshopper and Locust Control a Fair Chance

We know it can be done differently. The Australian government made a clear-cut decision to support the development of a similar product, Green Guard™ for the control of Australian plague locust (*Chortoicetes terminifera*) (Hunter *et al.*, 2001). The programme was carried out by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and supported from the outset by the Australian Plague Locust Commission. The incentives were different. Australian plague locusts were invading land belonging to cattle ranchers that produce high-priced, organic-labelled beef for the Japanese market. Australia is a large country and the producers of Green Guard™ only had to deal with a single national authority. Registration was relatively easy in terms of cost and time for a product with a 'green' image. Finally Australia is a developed country that can finance and support such a programme in a sustainable way without donor resources.

Microbial products such as Green Muscle™ can never be a 'silver bullet' solution for pest control. Its properties make it a viable product for preventative grasshopper and locust control, with some limitations for effective use under extreme temperature environments. The FAO's pesticide referee group specifically recommends Green Muscle™ for applications in environmentally sensitive areas,

where the use of conventional pesticides can be particularly detrimental. Preventive desert locust control was successfully implemented during the 1986–1989 desert locust plague in the Red Sea region, but because of the absence of efficient regional organizational structures in the West Africa region, preventive control failed in Mauritania, Senegal, Morocco and Mali, leading to the 2004 outbreak (FAO, 2004).

As part of an integrated desert locust control strategy, *Metarhizium* can play an important role in preventive treatments, particularly when coupled with accurate forecasting, which is now possible through the use of Geographic Information Systems (GIS).

Based on GIS data, the Australian Plague Locust Commission (APLC) claims to be able to very accurately predict locust hatching in time and space through the development of a Decision Support System (DSS). This computer-based system integrates weather data with current and historical information on locusts obtained from locust surveys, reports and light-trap records. Mathematical models are used to predict the development and survival of locust life stages and to forecast where migrations are likely to occur during favourable weather conditions. Such systems could also be adopted to define the spatial and temporal opportunities where *Metarhizium* could be used to best effect.

In addition to microbial control, desert locust and grasshopper IPM can benefit from utilizing additional biological tools in a combination approach. However, we believe that chemical insecticides will still play a dominant role in locust control at least until some of the key limitations to the biological approach discussed in this chapter are overcome. To create a sustainable economic basis for Green Muscle™, markets outside Africa should be explored, particularly in developed countries, where purchasing power and crop values are higher, where customers are less dependent on subsidies or international donor agencies and where market opportunities are stable.

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35

Pollinators as Vectors of Biocontrol Agents – the B52 Story

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Overview: Pollinating and flower-visiting insects can carry some plant diseases and can themselves be infected while foraging at flowers. This is the story of the development of the concept that pollinators could be used as carriers and disseminators of microbial biocontrol agents.

Considerations of How to Apply Symbioses to Pest Control

It is quite well known that pollinating and flower-visiting insects can carry some plant diseases and are themselves infected by diseases while foraging at flowers. Two of the best-known examples of pollinator-borne plant diseases are *Ustilago violacea*, the anther smut of various species of the pink family (Caryophyllaceae), and mummy berry (*Monilinia vaccinii-corymbosi*) on species of blueberry (*Vaccinium* spp. (Ericaceae)). Throughout Kevan's interest in insect and flower interrelationships has been an appreciation of such broader and more complex interactions. Thus, when research on pollination biology in field milkweed (*Asclepias syriaca*), in collaboration with D. Eisikowitch from Tel Aviv, ran into unexpected complications, the issue of tri-kingdom symbiosis arose. From that started our considerations of how to apply such symbioses to pest control.

The milkweed problem came about because Eisikowitch and Kevan were unable to germinate the pollinia in milkweed nectar collected from field-opened flowers, but could do so with full success in sugar solutions prepared in the laboratory or from nectar from flowers that had opened in the laboratory. The explanation seemed to reside in the yeast infections by *Metschnikowia rekaufii* in the flowers of field-opened flowers. The presence of the yeast inhibited pollinial germination (Eisikowitch *et al.*, 1990)! Review of old literature revealed that this yeast was known to be vectored by various flower-visiting insects. Other colleagues became involved in the work, notably Andre Lachance from the University

of Western Ontario, who has since made major taxonomic and ecological discoveries about *Metschnikowia* and its relations with insects and flowers.

Because of the capacity of *M. reukauffii* to interfere with pollinial germination in nectar, which in milkweeds is the germination medium and secreted by the stigmatic surface, Kevan and colleagues suggested that artificial application could inhibit sexual reproduction and seed-set in this important weed. Initial experiments were made, but before proper and full testing could be completed, funding was discontinued and research on potential application stopped. It is interesting that the genus (i.e. *Metschnikowia fructicola*, the 'killer yeast') has been tested as an inhibitor of grey mould on tender fruit (Kurtzman and Droby, 2001; Karabulut *et al.*, 2003) but not by using insect vectors for its dissemination.

Can Pollinators Provide a Double Benefit of Combined Crop Pollination and Protection?

The idea that biological control agents could be dispersed by flower-visiting insects is hardly new if one considers that pollination itself involves the dispersal of the biological control agent of fertilization (pollen) for most flowering plants. However, that idea, combined with knowledge that some important plant diseases (e.g. grey mould, fire blight, mummy berry) were also vectored by pollinators, stimulated research into the possibility of the double benefit of combined crop pollination and protection. The B52 project, to use honeybees (*Apis mellifera*) as vectors (bombers) for *Clonostachis roseum* to the flowers of strawberries (*Fragaria x ananassa*) to suppress grey mould (*Botrytis cinerea*), was initiated from John Sutton's laboratory, spearheaded by Peng Gang in collaboration with Kevan's group (Peng *et al.*, 1992). The dispensers used were made by adapting the design of the Nova Scotia Agricultural College Pollen Dispenser, developed for pollination in pome crops (King and Burrell, 1933). That work was followed by parallel studies with raspberries (*Rubus idaeus*), and using honeybees and bumblebees (*Bombus impatiens*) to bomb flowers with *C. roseum* (Yu and Sutton, 1997). The dispenser for bumblebees is a small box mounted to the hive entrance so that the bees have to walk a zig-zag track past baffles and through the inoculum before being able to fly out of the hive (Fig. 35.1). Again, the device directs outgoing bees through the inoculum, but incoming bees enter the hive by a different route isolated from the inoculum.

The results of the trials indicated that bumblebees, like honeybees, were as effective in vectoring the inoculum of the antagonistic fungus and suppressing the incidence of grey mould as was spraying fungicide at the normally recommended rates. The incidence of flowers with no inoculum was high in plots sprayed with the fungus (55–57%) compared with plots treated by bumblebee- or honeybee-vectored fungus (6–9% and 14–15%, respectively) (Yu and Sutton, 1997). Despite the levels of initial success in fruit protection matching or exceeding those achieved with conventional fungicidal sprays, funding was unexpectedly terminated. Since that time, the use of *C. roseum* has been promoted and used in various parts of the world for the protection of various tender fruits and other crops (Sutton *et al.*, 1997).

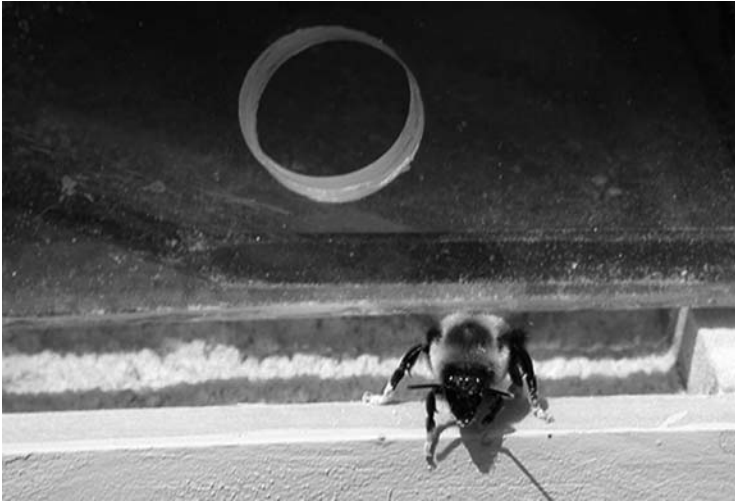


Fig. 35.1. A bumblebee sitting on the edge of a dispenser coated with inoculum.

Using the same technology as described above, a commercial formulation of *Trichoderma harzianum*, another antagonist to grey mould, has been applied to strawberries by pollinating honeybees in Italy (Maccagnani *et al.*, 1999) and by honeybees and bumblebees in the USA (Kovach *et al.*, 2000). The conclusion from that work is that bee delivering of *T. harzianum* is also a viable option for strawberry growers interested in controlling grey mould with minimal use of fungicides.

Further advances made at the University of Guelph were the testing of various carrier/diluent agents for the biological control materials. Israel and Boland (1993) found that some carriers, such as talc and especially scented talc, were irritating to the bees, which groomed much of the formulation from their bodies. Other carriers, such as flours, were better accepted by the bees and resulted in more efficacious transport of the agent. Israel and Boland's interests were for the suppression of *Sclerotinia* on the flowers (anthoplane) of rape (*Brassica* spp.), but application of the technology to that system remains to be taken up again. The results of their diluent/carrier tests have been important in the authors' recent research programme (see below).

At about the same time as the B52 project was proceeding, research in the western USA by Sherman Thomson's team in Utah (Thomson *et al.*, 1992) and Kenneth Johnson's team in Oregon (Johnson *et al.*, 1993a,b) was directed at using honeybees to deliver the bacterium *Pseudomonas fluorescens* as an antagonist against fire blight *Erwinia amylovora* on pome crops. That research also met with some success, has continued at a modest pace (e.g. Nuclio *et al.*, 1998) but recently has excited some renewed interest in Washington (Pusey, 2002).

An exciting development by Harry Gross and his team at the USDA laboratory in Tifton, Georgia was the first application of the B52 idea against insect pests (Gross *et al.*, 1994). Honeybees were used to deliver *Heliothis* nuclear polyhedrosis virus (NPHV) to crimson clover (*Trifolium incarnatum*) to help control *Helicoverpa zea*, the corn earworm (Lepidoptera: Noctuidae). The dispenser

developed for this project is elaborate and commanded its own patent. That initiative seems not to have been followed up, again despite its potential for practicality. Nevertheless, Tariq Butt and his group, working in Rothamsted, UK, revitalized the idea of using the system to control insect pests when they applied *Metarhizium anisopliae* to the flowers of rape (*Brassica napus*) to suppress populations of pestiferous pollen beetles (*Meligethes aeneus*) (Butt *et al.*, 1998). They used a modified Nova Scotia Agricultural College Pollen Dispenser and demonstrated efficacy of control. Shortly after that, research in North Dakota by J.L. Jyoti and Garry Brewer (1999) demonstrated that honeybees could be used as effective vectors of *Bt* (*Bacillus thuringiensis* var. *kurstaki*) to the flowers of sunflower (*Helianthus annuus*), where control (equivalent to that obtained with manual sprays) of banded sunflower moth (*Cochylis hospes* (Lepidoptera: Tortricidae)) was achieved, along with increased pollination and seed-set.

Our research on using B52 technology against insect pests started in response to the outbreak of tarnished plant bugs (TPB) (*Lygus lineolaris*) on rape in Alberta in 1998. On rape, adult TPB and late-instar nymphs cause direct yield loss by feeding on individual seeds through the pod (silique) and indirect loss by feeding on other plant tissues. TPB are important pests of numerous crops, including those grown in greenhouses. So, when Mohammad Al-mazra'awi joined Kevan's laboratory for his doctoral studies, an expanded study that embraced the biocontrol of TPB by the B52 approach on rape as a field crop and sweet peppers as a greenhouse crop was initiated. On greenhouse sweet peppers (*Capsicum annuum*), TPB causes yield loss by feeding on the growing points of the plant as well as on the developing flower buds and reproductive structures. As the project unfolded, the potential for control of western flower thrips (WFT), *Frankliniella occidentalis*, became evident. WFT is also a major pest of greenhouse sweet pepper and causes direct yield loss by feeding on, or ovipositing in, developing fruits, which results in a bronzing and silvering of the fruit. Previous reports had already demonstrated the susceptibility of TPB and WFT to the entomopathogenic fungus *Beauveria bassiana* (Bidochka *et al.*, 1993; Gindin *et al.*, 1996), known to cause mortality to TPB through disintegration of the insect cuticle and muscle tissues (Bidochka *et al.*, 1993), so it has been the entomopathogen central to our investigations.

Are the Entomopathogens Safe for the Pollinators?

One of our concerns has been the safety or the possibility of killing the messengers (bombers) with the biocontrol fungus. The latter also involves the nature of the diluent/carrier and the dispenser design (see Bilu *et al.*, 2004 for recent discussion of dispensers).

Safety tests can be made in various ways, in the laboratory with caged pollinators and in experimental hives. The biological control agents so far tested seem safe for honeybees and bumblebees if used at appropriate concentrations. Even entomopathogenic agents have been shown to be safe, except in the extremely high concentrations as in the commercially sold formulations of powders or liquids (Vandenberg, 1990; Alves *et al.*, 1996; Al-mazra'awi, 2004). For example,

we have found that we need to dilute Botanigard 22WP7, a formulation of *B. bassiana*, from 2×10^{11} conidia/g of product to 6×10^{10} to achieve minimum mortality of the bees and maximum mortality of the pests. At that concentration, *B. bassiana* has little effect on honeybees and it would not be expected to survive in the heat of the brood chamber (ca. 35°C) of the hive. It seems that bumblebees are a little more susceptible to developing mycosis from *B. bassiana* but that the risks are small.

In developing our formulation, we first evaluated factors affecting the acquisition of conidia of *B. bassiana* by honeybees in the laboratory using inoculum dispensers that allowed the bees to walk through different formulations of the agent. The number of conidia carried by bees emerging from the dispensers differed according to the type of formulation used. Honeybees that passed through maize flour acquired more conidia (e.g. 1.5×10^6 CFU (colony forming units)/bee) than did bees that passed through wheat flour, durum semolina, maize meal, potato starch, potato flakes, oat flour and barley flour. We find that 2 g of 6.24×10^{10} conidia of *B. bassiana*/g of formulation in dispensers on hives of 50 workers of *B. impatiens* minimizes risk to the bees while optimizing pest control. As a general rule, we found that the density of conidia carried by the bees increased with decreasing particle size and moisture content of the carrier and with increasing density of *B. bassiana* conidia in the formulation. Time required for honeybees to pass through the dispenser did not significantly affect the acquisition of conidia. After those trials, we chose maize flour as our favoured diluent/carrier. All in all, and although we continue to refine our formulation, it is evident that bees walking through such diluted inocula become sufficiently dusted with spores to control pestiferous populations of TPB on rape (Fig. 35.2) and WFT on greenhouse-grown sweet peppers, as well as green peach aphid (*Myzus persicae*), also on the latter.

Are Pollinators Efficient in Delivering the Biocontrol Agent?

Our second concern has been the efficacy of delivery by pollinators to field (i.e. honeybees, *A. mellifera*) and greenhouse crops (i.e. bumblebees, *B. impatiens*).

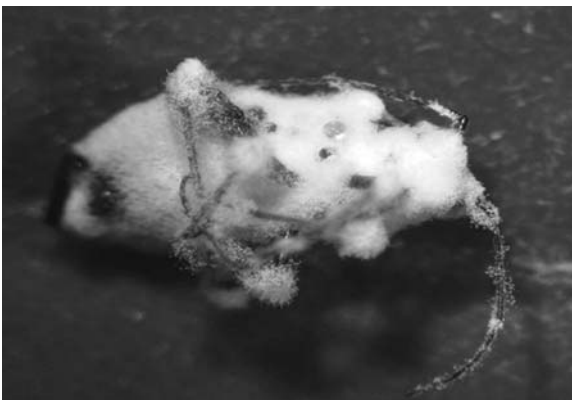


Fig. 35.2. A dead lygus bug killed by *Beauveria bassiana*. The fungal inoculum was brought to the host by a bee.

To test the dissemination of *B. bassiana* to rape against TPB, we used honeybees from nuclear hives equipped with inoculum dispensers (as developed for use with *C. roseum*) filled with our developed dry formulation of *B. bassiana*. The bees were allowed to forage on blooming rape plants inside large screened cages ($1.8 \times 6 \times 1.8$ m high) in a greenhouse and in open field plots. Samples of honeybees, flowers, leaves and TPB were collected from the cages on two sampling dates separated by 6-day intervals. The samples were subject to serial dilution plating to determine densities of *B. bassiana* conidia. TPB adults were also collected from the cages to assess mortality over time. Results showed that the bees effectively vectored the inoculum from the hives, as conidia of the fungus were recovered from 100%, 67% and 77% of bees, flowers and TPB, respectively, collected on the first sampling date, and from 100%, 77% and 83%, respectively, on the second sampling date in 2002. In 2003, 100%, 64%, 70% and 47% of sampled bees, flowers, leaves and TPB had *B. bassiana* conidia on the first sampling date, and 100%, 72%, 82% and 57% on the second sampling date. Mean mortalities of TPB collected from treated rape plants in the greenhouse trial were significantly higher (37%) than of TPB collected from the control plants (18%). In the open field trials, mean mortalities of TPB collected from the treated rape were 56% and 48% compared with 9% and 10% in the controls on the first and second sampling dates in 2002, and 22% and 45% in the treated rape compared with 15% and 22% in the controls on the first and the second sampling dates, respectively, in 2003.

Our group has tested, and is testing, the ability of bumblebees to disseminate conidia of *B. bassiana* from hive-mounted dispensers (noted above) to the flowers of greenhouse sweet pepper for the control of TPB and WFT. Evaluations were made inside large screened cages ($1.8 \times 4 \times 1.8$ m high) placed inside a greenhouse. Our most recent results, from samples collected from the cages on two dates, showed that 97% of the collected bees, 90% of the flowers, 91% of the leaves and 42% of the collected TPB showed detectable densities of *B. bassiana* on the first sampling date. On the second sampling date, 99%, 96%, 87% and 30% of collected bees, flowers, leaves and TPB showed detectable densities of the fungus. Mean mortalities of TPB collected from plants treated with *B. bassiana* were significantly higher (34% and 45%) than in the controls (9% and 15%) on the first and the second sampling dates. Mean infection rates of WFT collected from treated plants were 40% and 35% compared with 3% and 2% in the controls on the first and the second sampling dates. Our research is continuing with trials against green peach aphid and greenhouse whitefly (*Trialeurodes vaporariorum*) on greenhouse crops, including tomato.

Discussion and Conclusions

Developing a pollinator–vector technology for the management of insect and fungal pests on field crops, such as rape, and on greenhouse crops, such as sweet pepper, brings the benefits of reducing pest populations and pesticide use while improving pollination of the crop.

Insect pollination of rape is necessary for high seed germination rate, higher seed-set and yields. Similarly, using bumblebees for the pollination of

greenhouse sweet pepper resulted in increased fruit weight, volume, seed weight and percentage of extra-large and large fruits and reduced the number of days to harvest. Both honeybees and bumblebees effectively vector biocontrol agents such as *C. roseum*, *T. harzianum*, *B. subtilis* and *P. fluorescens* against plant pathogens and *B. bassiana*, *M. anisopliae*, *Bt* and NPHV to field and greenhouse crops and to orchards, where the populations of the various pests have shown to be reduced (review in Kevan *et al.*, 2003). This is a win-win situation because the pollinator vector technology not only reduces pest pressure and pesticide applications but also improves pollination. However, for this technology to be practical, the biocontrol agent must be shown to be safe for the bees. Laboratory tests followed by monitoring of the colonies during and after the trials must both show no adverse effect on the bees. The development of appropriate formulations and dispensers are key considerations for the success of the pollinator vector technology (Kevan *et al.*, 2003). The mix of the dry infective propagules of microbial control agents with diluent/carriers must be made with care to maximize safety and bee dissemination. Well-formulated agents have extended use in the field, and adjustments provide flexibility in preparing inocula with different concentrations to maximize the cost:benefit ratio. We caution that trials are needed for each combination of biocontrol agent and its formulation, the type of pollinator used, the crop to be protected and the pest targeted by the technology, and the sort of dispenser considered to be the most appropriate. Thus, pollinator-vector technology is a multi-disciplinary pest management approach that incorporates different ecosystem components such as pollinators, microbial biological control agents and insect pests in crop production. It brings the benefits of pest management, reduced chemical use and better pollination for the crop, which subsequently results in higher yields and better crop quality.

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36 Genetic Modification for Improvement of Virulence of *Metarhizium anisopliae* as a Microbial Insecticide

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Overview: Biocontrol experiments with fungi have often produced inconsistent results and this has deterred commercial development. However, many fungi are amenable to genetic modification for purposes of enhancing utility for disease control, insect and plant pest management or bioremediation. In such cases, genetically engineered fungi may provide environmentally preferred alternatives to current chemical-based control strategies. This is the story of the first genetic manipulations of an entomopathogenic fungus, *Metarhizium anisopliae*, in an effort to improve its potential as a microbial biopesticide.

The Selection of a Strategy

A basic problem confronting anyone considering genetic improvement of biological control agents is how to alter their genetic make-up to improve their effectiveness as biocontrol agents without producing a hazard to non-targets (St. Leger and Screen, 2001). In general, pathogens can be made more effective by increasing rate of reproduction, speed of transmission and infective ability, or increasing the quantity of toxins produced.

The ascomycete entomopathogen *Metarhizium anisopliae* is widely applied abroad, was recently registered for use in the USA and Europe and offers particular promise as a suppressive agent for many soil insect pests that would otherwise provide a particular challenge to pest control specialists; thus it seemed to be the ideal candidate for genetic improvement. But what to change? We opted for increasing production of toxins and enzymes in our studies. This was mostly for practical reasons. Insect pathogens secrete a plethora of enzymes and toxins that show pathogenic specializations symptomatic of millions of years of evolution refining chemicals that subdue their hosts, so one is not spoilt for choice. Also, since many of these enzymes/toxins are encoded by single genes they are highly amenable to genetic manipulation. In addition, we felt that by using a gene derived from the pathogen itself we would be less likely to raise public concern than trying

to release a fungus for pest control that carried a gene from something with an intrinsically frightening name like virus.

Results Are Not Always As Expected

The addition and expression of pesticidal genes in *M. anisopliae* is quite straightforward. In our best-studied example, additional copies of the gene encoding the regulated cuticle-degrading Pr1A subtilisin protease were inserted into the genome of *M. anisopliae* under control of the *gpd* promoter such that the gene was constitutively overexpressed (St. Leger *et al.*, 1996). At the commencement of this experiment, our expectations were that if overexpression of a cuticle-degrading protease were to have an effect on pathogenicity, it would be to speed up penetration. However, the actual effect of overexpression was to cause massive melanization within the body cavity (Fig. 36.1) and cessation of feeding 40 h earlier than controls infected with wild type. We found that, unlike the wild type, transgenic strains continued to produce Pr1 in the haemocoel following penetration of the cuticle. This activated a host trypsin-like enzyme that is involved in a cascade terminating in prophenoloxidase activation.

Our microarray experiments have since shown that *M. anisopliae* stops expressing protease genes in haemolymph, presumably to prevent this from happening (Wang *et al.*, 2005). Thus was initiated a trend in most of our engineering studies. They hardly ever turn out as we predict. We may know in considerable detail the properties of an individual gene and its protein product. We may know



Fig. 36.1. Insects killed by bioengineered (above) and wild-type pathogen (below).

how it interacts with the other proteins produced concurrently with it in the native organism. However, transfer it to another organism and the results are unpredictable because they involve unforeseen interactions with proteins that it would not normally interact with. In the particular case of a pathogen, it is even harder to predict consequences because there will be interactions between the protein products of two genomes, as with the pathogen protease and the host phenoloxidase. In recent years, we have largely given up on our previous hypothesis-driven approach of cloning of individual genes implicated in pathogenicity, as it did not provide much understanding of interrelated regulatory and metabolic processes going on in the cell and between the host and pathogen. Instead, we focused on cost-effective expressed sequence tag (EST) approaches and constructed microarrays to 'look under the boot' of the pathogen and let it tell us what it is doing during infection processes (Freimoser *et al.*, 2005; Wang and St. Leger, 2005; Wang *et al.*, 2005). Once the data are in, then we generate hypotheses to test the contributions to pathogenicity of individual genes that are sharply up-regulated during some phase of the infection processes. This approach has revealed previously unsuspected stratagems of host-pathogen interactions. For example, the most highly expressed gene during growth in haemolymph turned out to be a cell wall protein with a long collagenous domain (5.6% of total transcripts). Immunofluorescence demonstrated that the collagen coats the fungus during growth in haemolymph, and gene knockout confirmed that it is required for immune evasion (Fig. 36.2). The mutant is rapidly attacked by haemocytes and has reduced virulence to *Manduca sexta*. RT-PCR confirmed that *Mcl1* is expressed during growth in the haemolymph of a diverse array of insect species, consistent with the broad host range of 2575. However, it was not expressed in other media, consistent with it being involved in pathogenesis (Wang and St. Leger, 2006). These results emphasize that the effectiveness of pathogens as biological control agents will be determined by the efficacy of the insect's immune system. Thus, fungal adaptations to host defenses are likely to play an important role in virulence and specificity.

Seeking Regulatory Approval for Field Release

The subtilisin overexpressing strain had several features that we thought were promising in a candidate for a field trial and we consulted with the Biopesticides and Pollution Prevention Division of the EPA Office of Pesticide Programs. They quickly informed themselves about the fungus and its safety record. So by our first meeting, they knew that instances where *M. anisopliae* had been associated with human disease (e.g. Cepero *et al.*, 1997) were very rare, even when compared with the most widely used biocontrol agent, *Bacillus thuringiensis*, or that most domesticated of microbes, *Saccharomyces cerevisiae* (often referred to as brewer's or baker's yeast). In fact, excepting vaginitis, there have been 22 cases of clinical infection by *S. cerevisiae* reported in the English language literature, with 50% mortality (Fiore *et al.*, 1998). Given the many years of extensive pathogenicity/toxicity testing with *M. anisopliae* (e.g. Goettel *et al.*, 1990), they concluded that there are few if any health risks associated with use of this fungus.

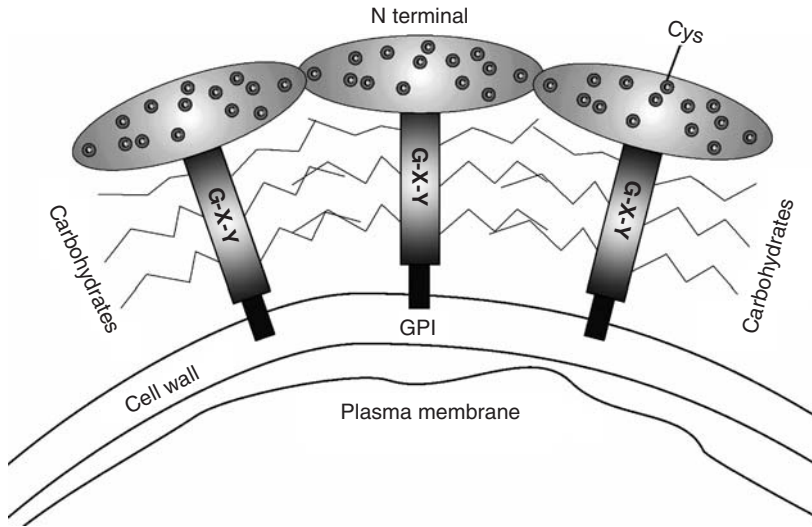


Fig. 36.2. The pathogenic fungus *Metarhizium anisopliae* evades host immune responses using a unique protein that comprises an anti-adhesive N-terminal domain held above the cell surface by an extended collagenous (G-X-Y) fibre (Wong and St Leger, 2006).

First Some Preliminary Laboratory Studies

Following our discussions with the EPA, we did a set of preliminary laboratory studies. First, while it killed faster, the LD₅₀ had not changed and we found no evidence for altered host range. This result was predictable as the efficiency of infection (attachment and penetration) should be unaltered by continuance of Pr1 production after cuticle penetration. For most insect pathogens, specificity is determined by events at the cuticle. If the fungus succeeds in penetrating that barrier, it is usually just a matter of time before the host dies. However, for the EPA, it was a critical consideration as obviously you would not want to release in the field a pathogen that could kill arthropods that mankind deemed to be useful. Secondly, while the development of pathogens for classical biological control depends on their being able to recycle through host populations, most pathogens currently being considered for genetic enhancement would be applied in an inundative manner, and there are both environmental and economic reasons why you would not want an engineered pathogen to persist in the field. However, the melanized cadavers of insects killed by the transgenic fungi were very poor substrates for growth and reproduction of the fungus. This suggested a degree of biological containment because of reduced potential for secondary infection. However, because of the great complexity of environmental impacts, it is impossible to mimic nature indoors and extrapolate lab data to a field situation, and unfortunately we know very little about the survival of individual genotypes (clones) of entomopathogenic fungi in nature. Thus, although the transgenic *M. anisopliae* does not sporulate on host cadavers, that does not mean that it could not survive in a soil or plant

environment. Furthermore, we were using a strain unrelated to the great majority of North American isolates, on the premise that this would provide barriers to anastomosis-mediated parasexuality and the spread of the transgene to native populations. However, there is no experimentally derived information on gene transfer from populations of genetically engineered pathogenic fungi to wild-type or other fungal species.

Field Releases

Regulatory bodies such as EPA, as well as scientists themselves, are seeking systems that balance relative risks with relative benefits, and to achieve successful, reproducible and safe (from the risk-management point of view) biological control, we need to be able to study the ecology of the transformed genotype. We were consequently granted approval (38567-NMP-R) from EPA to conduct a release on a plot of cabbages at a field site on the Upper Marlboro Research Station, Maryland (Hu and St. Leger, 2002). The approval constrained us to establishing the technology required to follow the fate of genetically enhanced *M. anisopliae* and to using this technology to determine the potential of engineered strains to establish and disperse over a 1-year test period. The transformed derivatives of *M. anisopliae*, designated GPMa and GMa, carried the *Aequorea victoria* green fluorescent protein (*gfp*) gene alone (GMa) or with additional protease genes (*Pr1*) (GPMa). A major point of the study was to monitor fate (survivorship) of transformants under field conditions.

The Importance of the Rhizosphere

Since root exudates stimulate the growth of many bacteria and fungi in soils, and the rhizosphere (root/soil interphase) is of great importance to plant health and fertility, we focused on it as a potential refuge for transgenic fungi, which could increase their persistence. In non-rhizosphere soil, GMa decreased from 10^5 propagules/g at depths of 0–2 cm to 10^3 propagules/g after several months. However, densities of GMa remained at 10^5 propagules/g in the inner rhizosphere, demonstrating that rhizospheric soils are a potential reservoir for *M. anisopliae*. These results place a sharp focus on the biology of the soil/root interphase as a site where plants, insects and pathogens will interact to determine fungal biocontrol efficacy, cycling and survival.

However, the rhizospheric effect was less marked for GPMa, and overall it showed reduced persistence in soils compared with GMa. This reduced fitness and survivability could reasonably derive from deleterious effects of the additional genetic modifications, as compared with transgenic fungi expressing GFP only. If so, then *M. anisopliae* may not just persist in the soil in a dormant state, but characteristics for soil survival may include gene expression that can be interfered with by plasmid integration. The preferred natural habitats of common entomopathogens such as *Metarhizium* and *Beauveria* spp. remain unclear. However, *Beauveria bassiana* has been demonstrated to be a leaf endophyte, functioning as a

protective mutualist (Wagner and Lewis, 2000). Persistence of *M. anisopliae* in the rhizosphere suggests that associations with plants may be a general phenomenon among mycopathogens, with implicit co-evolutionary implications and impact on plant ecology. Although this study does not show that growth of *M. anisopliae* has occurred in the rhizosphere, population levels in the inner rhizosphere remained constant while most other studies using fungi that are known to be good root colonizers show a decline, perhaps because the initial population added is too large for the carrying capacity of the root (Parke, 1991). At the least, soil in the vicinity of living and dead plant roots provides a refuge from factors in the environment that otherwise reduce the titre of *M. anisopliae*.

Rhizosphere competence is particularly important when considering the potential commercial use of biocontrol agents towards soil-borne plant pathogens, and presumably the same could apply to pathogens of root insects. Most studies employing the facultative saprophyte *M. anisopliae* have selected strains for optimum virulence against pest insects and have ignored habitat preferences and survival outside the host. The search for highly virulent isolates of this fungus may be inherently flawed, given that factors associated with soil dwelling may be even more critical in the selection of an isolate than virulence per se (Bidochka, 2001). Thus, in the case of strains engineered for improved virulence, such strategies may fail if genes are engineered into a strain that survives poorly in a certain habitat.

Rhizosphere competence also raises the possibility of managing the rhizosphere microflora to achieve insect control. If a good root colonizer that is capable of being transported by the root through the soil profile is chosen, then seed treatment would be an attractive method for introducing it into the soil-plant environment, where it may have the opportunity to be the first colonizer of roots. On the other hand, rhizosphere competence might increase the difficulty of eliminating the pathogen following unanticipated and deleterious environmental effects.

Some Tentative Conclusions

The risks we evaluated during this trial allow us to produce some tentative conclusions as to the safety of employing GMa and GPMA in field conditions (Hu and St. Leger, 2002). Soil analysis before and after field release showed that the profile of indigenous culturable fungi was not affected by the field trial application. With the caveat that not all fungi are culturable, these results indicate that there is minimal risk of the engineered fungus displacing naturally occurring fungi. Given the impoverished fungal microflora observed in the cultivated compared with the non-cultivated (fallow) land, we expect that the impact of introduced microorganisms in general will be only minor compared with common agricultural practices such as plowing or crop rotation. We found only flea beetles and *P. rapae* among non-target insects carrying spores. These results suggest the potential of insect-mediated dispersal to non-targeted deployment areas is low and there was no evidence for an increase in host range reflected in infected beetles, ants, etc. No infected insects were found outside the application area, so unassisted spread of the pathogen is also likely to be slow. However, the survival

of both strains is significant as it will provide time for dispersal and also provide the opportunity for the pathogens to change with respect to environmental conditions and time.

There was no evidence for phenotypic instability of the introduced fungus, i.e. it had not apparently diversified from the input transgenic strain to produce different types. However, this would be expected if genetic changes visible as growth patterns, conidiation, etc. follow the punctuated equilibrium model of evolution, with long periods of apparent stability, punctuated by large infrequent changes. It is an unsatisfying and inconclusive generalization, but the long-term fitness of a genetically engineered pathogen in nature is difficult to predict with much accuracy from statistical analyses, controlled experiments or field trials. However, now that we have established the technology that will at least permit informed risk assessment through following the fate of marked strains, we will hopefully be able to perform more extensive quantitative trials with second-generation transgenic fungi. We are in the process of producing transgenic hypervirulent *M. anisopliae* that express much more acute toxins than Pr1A, including a Phospholipase A1 and the 70 aa AaIT neurotoxin from the scorpion *Androctonus australis*. We also intend to engineer these with modifications designed to reduce survival in the field.

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37 Madex[®] and Virosoft^{CP4®}, Viral Biopesticides for Codling Moth Control

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Overview: This chapter relates the research and development of two granulovirus-based insecticides to manage the codling moth, *Cydia pomonella*, which is a worldwide pest of a number of fruit crops. Madex[®] and Virosoft^{CP4®} were registered in Switzerland and Canada, respectively.

The Codling Moth

The codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), is a worldwide pest of a number of crops, including apples, pears and walnuts. Its literature is considerable, as the Codling Moth Information Support System indexes ca. 6500 references (CMISS-2005). Standard commercial management practices consist of several sprays per season of synthetic pesticides applied against larvae. Among alternatives to insecticides, parasites and predators have been studied but their effects are below expectations in commercial situations. Mating disruption has been successfully implemented in several apple-producing regions. However, this method is relatively costly and needs an environmentally friendly back-up method in case of failure.

The Baculovirus

Baculoviruses are DNA viruses that naturally infect arthropods, chiefly insects. One of their features is that they infect only larval feeding stages, in which they form proteinaceous structures called occlusion bodies (OBs). A second feature is that many baculoviruses contain multiple virions (genomes) in each infective OB.

Baculoviruses are classified into two groups, i.e. the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). The latter, characterized by rod-shaped virus particles occluded singly within a granular protein capsule or occlusion body, have only been reported in lepidopterans. Each OB (600–800 nm) contains a single virion enclosing one nucleocapsid (see Moscardi, Chapter 38 and Lucarotti *et al.*, Chapter 39 this volume).

The *C. pomonella* granulovirus (CpGV) was first found in apple and pear orchards growing near Chihuahua, Mexico (Tanada, 1964). The mode of action of CpGV is similar to that of other baculoviruses associated with lepidopterans. After ingestion, granulin is dissolved in the gut by the combined action of alkaline pH and proteases. Granules liberate virions, which penetrate the epithelial cells of the gut through the peritrophic membrane. When the infection cycle is completed, larval body tissue is converted into millions of OBs, and the cycle resumes through horizontal and possibly vertical infection. One outstanding characteristic of CpGV is its extremely high pathogenicity: its LD₅₀ is 1.2 granules for neonates.

CpGV is rather specific, although it may kill the oriental fruit moth, *Grapholita molesta* (Falcon *et al.*, 1968), it does not impact natural enemies (Jacques *et al.*, 1981; Glen *et al.*, 1984). As it was found to be highly virulent and amenable to commercial production, field trials were conducted in apple orchards of numerous countries, including the USA, Switzerland, Germany, Canada and the UK. These (and other) research projects paved the way to the registration of CpGV for agricultural use in several countries. At the time, there was a worldwide movement to develop and register viral insecticides for agricultural use.

The Development as Biopesticides

The codling moth granulovirus was commercially produced in 1980 as SAN 406 by Sandoz Inc. and exempted from a tolerance by US-EPA in 1981. Sandoz ended research and development in 1982 (Falcon and Huber, 1991). Several research and development projects were conducted in European countries in the 1980s (Falcon and Huber, 1991). Over the years, CpGV has been registered in a number of countries under the trade names Carpovirusine[®], Madex[®], Granusal[®] (now Granupom[®]) and Virin-CyAP (Moscardi, 1999). Recently, it has also been registered as Cyd-X[®] and Virosoft^{CP4®} (Arthurs and Lacey, 2004).

Because neonate larvae feed only for a short duration before they enter the fruit, timing is critical to prevent damage and to infect larvae when they are reachable by sprays. Codling moth neonates may ingest CpGV from both leaves and apple surfaces, and the amount ingested depends on exposure time and concentration of viruses. Formulation additives may increase the effect of CpGV in the field. For example, it has been shown in orchards that molasses significantly reduces the lethal exposure time required to kill larvae (Ballard *et al.*, 2000). However, it is unclear if molasses acts as a feeding stimulant or enhances the infection process. In temperate regions, the half-life of CpGV is 2–3 days (Ballard *et al.*, 2000).

This chapter relates the critical steps in the registration and marketing of two CpGV products. They were the first viral insecticide to be registered for agricultural use in their respective countries; they are produced by small companies

addressing small (niche) markets; and they are minimally formulated. Major differences are that CpGV was registered in Switzerland ca. 15 years before Canada, and that the ownership of the company has been consistently stable in Switzerland over the years whereas partnership between several organizations was pursued in Canada.

Madex® and the Birth of a Biopesticide Company in Switzerland

In the mid-1980s, organic apple producers of Switzerland had up to 50% fruit damage caused by the codling moth because there was no biocontrol product or method available against this key pest. As a student doing his MSc in biocontrol, one of us (M.A.) was aware of the literature on CpGV, as well as the huge discrepancy between the problem encountered in organic fruit production and commercially available solutions. I naively contacted the Swiss registration authority and asked why they registered only chemical products against the codling moth and not environmentally friendly products such as CpGV. The answer was simple: 'Bring us a registration file, we will check it, and if everything is fine, we will register such a product.' With the help of Prof. Jürg Huber, BBA Darmstadt, Germany, I put together a registration file, which was submitted in January 1987 to the registration authority in Switzerland.

In our three-room student flat, my wife Isabel and I produced a first batch of CpGV product *in vivo*, which was named Madex®. The artificial diet was cooked in the kitchen; the rearing of the healthy larvae was in one room, the virus infected larvae in another, and the bath-tub was used to wash the rearing units. Field trials conducted in 1987 gave good results. A provisional registration for Madex in Switzerland was granted in December 1987. This was a time when reasonably thinking people made reasonable decisions in a reasonable period of time! And all this without any fee!

Mass production and marketing were the next challenges. We moved in the same apartment house to a bigger flat because thousands of moths were needed to meet demand (Fig. 37.1). Isabel left her job as a veterinarian and produced more than 500 ha units of Madex at home. During a meeting, a small group of Swiss organic apple producers (pioneers) were informed of the availability of a new viral bioinsecticide. As these growers had no alternatives, they were easily convinced to try Madex and most producers bought some. Organic apple producers from neighbouring countries, i.e. Germany, Italy and France, also came to Switzerland to buy Madex in its very first year of production.

Andermatt BIOCONTROL AG was established early in 1988. After a first season, the company made a turnover of more than 50,000 CHF (ca. US\$ 40,000), a huge amount of money for a young couple! To establish a business and to become an entrepreneur seemed to be easy. After the birth of our first son, the apartment was again too small for the production and our family moved into an old farm building in Grossdietwil, a small village in the canton of Lucerne (Fig. 37.1). The rearing of codling moth was moved, as well as a stock of the leafroller *Adoxophyes orana* and its granulovirus. This was the basis to develop a second product named Capex®, with the support of Prof. Georg Benz of the Swiss



Fig. 37.1. Evolution and growth of the Andermatt facilities over the years.

Federal Institute of Technology, Zürich. In the same year, Andermatt BIOCONTROL started to produce insect parasitic nematodes (*Heterorhabditis megidis*) and soon other commercial biocontrol products and beneficial insects completed their product lines.

In the early 1990s, Madex was only used by organic farmers. Attempts were made in some European countries and in New Zealand to register Madex, but as the registration file was rather limited and the registration authorities were not used to registering such products, it took some years to get these registrations. As of May 2006, Madex was registered in 12 countries and many registrations were pending.

In the late 1990s the organic production boomed in Western Europe. At the same time, the resistance of the codling moth against most of the chemical insecticides became more and more obvious. Furthermore, many old active ingredients disappeared from the market. Suddenly, CpGV also became interesting for conventional producers, especially after Kienzle *et al.* (2002) demonstrated that even a single Madex application early in spring could reduce the overwintering codling moth populations by more than 50%. Apple growers accepted that a codling moth may enter a fruit before dying, but they knew that ultimately the larval mortality would be much higher than with many chemical insecticides. Finally, Madex is now an important IPM tool in several countries. The production scale had to be increased stepwise up to a capacity of over 250,000 ha units per year. The growth of Andermatt BIOCONTROL over the years is reflected by the growth of its facilities (Fig. 37.1).

Virosoft^{CP4®}: Producing CpGV in Canada and Registering It in North America

In Canada, the development of a CpGV-based biopesticide was achieved thanks to a partnership between the private sector (initially represented by BioTEPP and BioSag) and the Government of Canada. In 1996, infected codling moth larvae were collected in several regions of Quebec, Canada, and I (C.V.) bioassayed and screened at the Horticultural Research and Development Centre of Agriculture and Agri-Food Canada at St-Jean-sur-Richelieu. A highly entomopathogenic isolate showing a characteristic restriction enzyme nuclease pattern was selected for registration. A production plant was built in Cap Chat, Quebec, Canada, in 1999.

In 2000, BioTEPP registered its first product in Canada and the USA. The formulation, named Virosoft^{CP4®} contained 4×10^{13} occlusion bodies per litre and was applicable at a rate of 250 ml per ha. However, BioTEPP experienced both administrative and quality control problems. As a consequence, Virosoft^{CP4®} provided erratic effects in the field, which caused a loss of confidence by users.

In 2001, BioTEPP was acquired by Oligosol Ltée., a downsized and renewed organization, which undertook the challenge of regaining market confidence with a better-quality product. Major investments were made to redesign the Cap Chat facilities. Eggs were bought from the Okanagan-Kootenay Sterile Insect Release Program (OKSIR) in Osoyoos, British Columbia, and were flown weekly to Cap Chat for viral production. Improved quality control procedures were also implemented. Since 2003, field trials were conducted in a number of apple orchards in Canada (i.e. Quebec, Ontario and British Columbia) and in the USA (i.e. Washington State, New York State and Michigan). These trials confirmed that Virosoft^{CP4®} is a useful and reliable management tool for the codling moth.

Virosoft^{CP4®} preparations remain active on apple foliage for up to 14 days (Arthurs and Lacey, 2004). The virus survives longer on UV-protected substrates, such as the calyx of fruit. They also showed that Virosoft^{CP4®} stored at 35°C for 2 weeks and diluted at 1 : 1000 and 1 : 100,000 caused 100% mortality of neonates. The preparation diluted at 1 : 1000 and at 1 : 100,000 caused 100

and 93% mortality of neonates after storage for 13 weeks at 25°C and 35°C, respectively.

Thanks to the continuous improvement efforts and investments over the years, Virosoft^{CP4®} has regained the confidence of the scientific community, as well as major agricultural players in North America, as evidenced by scientific publications (e.g. Arthurs and Lacey, 2004) and agreements with major North American wholesalers and sales achieved in early 2005. Work is in progress to develop more efficient rearing methods and new formulations.

Lessons Learned

Madex and Virosoft^{CP4} were the first viral insecticides produced and registered for agricultural use in Switzerland and Canada, and this paved the way for the registration of other viruses for plant protection. In retrospect, several factors facilitated their registration: the relevant science (knowledge) and technologies (know-how) were ripe in the 1980s; the CpGV registered in Switzerland and Canada were naturally occurring (i.e. non-transformed), and this attribute probably facilitated registrations with minimal data; the market for codling moth is worldwide and the demand is continuous; the relevant social context, i.e. the emergence of 'green' markets in Western countries, especially in Western Europe, created a demand for biocontrol products such as CpGV-based biopesticides; and the codling moth developed resistance to several synthetic insecticides, notably in Western Europe in the late 1990s. Finally, compatibility with existing management techniques, such as mixture with reduced doses of chemicals or mating-disruption techniques, facilitated the demand (Charmillot and Pasquier, 2002). The latter authors concluded after a 7-year study in Switzerland that only combination of CpGV and mating disruption can sustainably maintain codling moth populations at low levels. With only CpGV treatments, these results can be achieved but with 4–6 treatments per season over several years (Pasquier and Charmillot, 1998).

Perspectives

It is possible that insect populations develop resistance to viruses under selection pressure, as discussed by Moscardi (1999). In Germany, two codling moth strains showing a much lower susceptibility to CpGV than all other strains have been found (Fritsch *et al.*, 2005). Recently, resistant populations have been found in southern France, where numerous applications per season have been made over several years (Sauphanor *et al.*, 2006). To delay the development of resistance, rotation with other control methods is advised. Several questions relevant to resistance or tolerance to CpGV remain to be answered, notably on the mechanisms involved. In the meantime, isolates have been found which overcome the resistance (Sustain CPGV).

Typical problems related to the use of viruses in the field, i.e. persistence on foliage, shelf-life and stability of formulation (the actual formulation of Virosoft^{CP4®} is sold and distributed frozen) must be addressed. One important, yet often overlooked,

aspect is the need to educate growers on the characteristics of viral insecticides (Moscardi, 1999, Chapter 38 this volume).

Now that CpGV has been completely sequenced (Luque *et al.*, 2001) and molecular techniques easily allow genotype transformations, a relevant question is: should a transformed CpGV be registered for agricultural use? Desirable attributes such as a shorter killing time and a greater potency are technically possible. In Europe, the registration hurdles for naturally occurring microorganisms and viruses are now on such a high level that additional questions related to toxicological and ecotoxicological issues concerning the use of such GMOs would be too expensive to answer.

The Holy Grail of viral insecticide production on a large commercial scale is *in vitro* production. *In vitro* replication of CpGV was first achieved by Naser *et al.* (1984) and further work was done by Winstanley and Crook (1993). Although advances have also been made in invertebrate cell culture (Maramorosch and Mitsuhashi, 1997), more work is needed to develop efficient and sustainable *in vitro* CpGV production on a commercial scale, which would allow for profound changes in the insecticide market worldwide.

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38

A Nucleopolyhedrovirus for Control of the Velvetbean Caterpillar in Brazilian Soybeans

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Overview: Implementation of an IPM project in Brazil to control the velvetbean caterpillar in soybean presented a unique opportunity to take advantage of a naturally occurring nucleopolyhedrosis virus against the pest. This is the story of the discovery and implementation of this virus, which is presently used on approximately two million hectares of soybean in Brazil, representing the largest programme worldwide for the use of an entomopathogen to control a pest in a single crop, which generated substantial economic, ecological and social benefits to Brazil.

Introduction

The velvetbean caterpillar, *Anticarsia gemmatilis* (Lepidoptera: Noctuidae), is a key pest of soybean in the Americas, ranging from northern-central areas of Argentina to the south-eastern USA. In Brazil, it is the most important defoliator in all soybean-producing regions, demanding an average of two insecticide applications for its control during the season. Up to the mid-1970s, most chemical insecticides used on soybean were organochlorides (such as DDT, endrin) and organophosphates (such as methyl parathion and monocrotophos), as well as mixtures involving both insecticide groups. At that time, concerns were raised among research organizations, such as Embrapa, regarding the overuse of such broad-range and highly toxic chemicals and their potential impact on man and the environment. This led to a pilot soybean integrated pest management (IPM) programme conducted in farmers' fields in different regions of Parana and Rio Grande do Sul states, during 1975 and 1976 (Kogan *et al.*, 1977). Techniques employed against major insects (*A. gemmatilis* and stinkbugs) were: (i) monitoring insect pest incidence periodically; (ii) applying selected insecticides at minimum efficient rates when the major insect pests reached action thresholds (as those used in south-eastern USA); and (iii) observing the incidence of major natural enemies of *A. gemmatilis*, such as the entomopathogenic fungus *Nomuraea rileyi*.

Results of this pilot work indicated that the average number of insecticide applications in soybean could be reduced by approximately 78% without any reduction of crop yield, compared with insect control initiatives used by soybean growers at that time. This new IPM programme was implemented among approximately 40% of soybean growers in subsequent years, facilitating the acceptance of biological control initiatives, which were introduced later (Moscardi, 1993).

In the 1970s, a nucleopolyhedrovirus of the insect (AgMNPV) was isolated in different regions of Brazil (Allen and Knell, 1977; Carner and Turnipseed, 1977), indicating a new perspective for controlling the insect. Initial field experiments with the AgMNPV indicated its potential to be used to control the insect in soybean IPM programmes (Carner and Turnipseed, 1977; Moscardi *et al.*, 1981). At the beginning of the 1980s, research results generated at the Brazilian Agricultural Research Corporation (Embrapa), National Center for Soybean Research (Embrapa Soja), in Londrina, state of Parana, were used to implement an AgMNPV pilot programme in soybean growers' fields (Moscardi and Corrêa-Ferreira, 1985; Moscardi, 1989, 1999).

Early (Pilot) Phase of the Programme at the Farmer Level; Seeing is Believing

A pilot programme for AgMNPV use was conducted under my coordination during the 1980/81 and 1981/82 seasons, on 21 farms in the southern states of Parana and Rio Grande do Sul, where 1-ha plots treated with the virus resulted in efficient control of *A. gemmatilis* populations compared with insecticide-treated and untreated paired plots at each location. During this pilot phase, an important aspect was the selection of farmers who were leaders in soybean production in each region. Officers of the official extension services were responsible for the selection of these farmers at each location and also for monitoring each pilot field and collecting data, such as larval populations, defoliation and yield in virus-treated, insecticide-treated and control plots.

At that time, all research and extension personnel involved were aware of the importance of using proper approaches to convince the soybean growers to use a novel control method, which was fundamentally distinct from the conventional chemical control (fast-killing against the slow-killing provided by the biological insecticide). Therefore, the approaches taken were basically the following: (i) talks by Embrapa Soja researchers to train extension officers on basic aspects of AgMNPV, viral dosage, timing of applications related to insect age and density based on weekly sampling, etc., to guarantee field efficacy; (ii) distribution of a folder containing all necessary information about how to use the AgMNPV; (iii) field days, involving Embrapa researchers, official and private (farmer cooperative) officers, and farmers of each pilot programme region, to show the paired areas and comment on the results (the farmer leader and the extension officer being made responsible for each site); and (iv) after each season under the pilot programme, Embrapa researchers and official extension

officers responsible for each site would meet to discuss the results and possible modifications to improve the programme.

In this pilot phase, there was clear reluctance of some of the selected leader farmers in even allocating the 3-ha area needed for testing the AgMNPV. I think it is important to mention one of the cases. In the Sertanópolis region, Parana state, I and a colleague from the official extension service visited the selected farmer to explain what had to be done. It was raining, and we were in a machinery storage building where the lead farmer was accompanied by some of his neighbouring soybean farmers. We showed them a plastic container with AgMNPV dead larvae and told them that those larvae were killed by a lethal viral disease that could be sprayed (after grinding with water and filtering through a cloth) on soybean plants to control the velvetbean caterpillar. The selected farmer and his neighbours started looking at each other and gave us a 'Mona Lisa' type of smile, the one that we interpreted as: 'We do not believe in what you are saying.' In any case, the selected farmer agreed to test the virus, according to our procedures, in respect to Embrapa and the local extension officer. Seven days after the implementation of this specific pilot area, I and other colleagues of Embrapa headed to this farm to evaluate the plots. On the way to the farm, on a dirt road, we encountered the lead farmer with a bunch of soybean plants in his hands. Some plants had low defoliation and many caterpillars hanging, killed by the AgMNPV, and others (taken from the untreated area) had only live caterpillars and high defoliation (over 50%). When we asked him what he was doing, he answered 'My neighbouring farmers were making fun of me all week about this biological thing to control the velvetbean caterpillar. I am going to a bar by this road to meet them and I will ask them to eat the dead caterpillars! This technology you are suggesting is very good. Thank you!'

Recognizing the importance of the AgMNPV technology was common among the other lead farmers involved in the pilot areas, and they were instrumental in convincing other farmers to change the paradigm of chemical use. Results were so favourable (Moscardi and Corrêa-Ferreira, 1985; also see Moscardi, 1999 and references cited therein) that Embrapa Soja and official and private extension services decided to formally implement a programme for use of the biological insecticide (AgMNPV) at the farmer level, initially in the south and later in other Brazilian soybean-producing regions.

Implementation/Consolidation of the Programme at the Farmer Level

Implementation of the programme began in the 1982/83 season, when ca. 2000 ha of soybean were treated. Initially, small amounts of AgMNPV were produced in host larvae reared on artificial diet at Embrapa Soybean facilities. Frozen killed larvae were distributed to extension officers for treatment of demonstration plots and virus production in the field, which provided inoculum to treat other areas in the same season or to collect and store dead larvae for the subsequent season. Before or during the 1982/83 season (October to early December),

researchers of the Entomology sector of Embrapa Soja travelled to different locations in the south, presenting talks about the most important aspects of AgMNPV use, the virus production in the field and storage of the material. Enough material was provided to treat 10 to 30 ha at each site, depending upon the number of farmers involved. Sets of slides were prepared for the presentations, as well as simple publications (leaflets, folder) for distribution at each location.

Virus use gained momentum with the development of a wettable powder formulation in 1986 (Moscardi, 1989, 1999). The Employees' Association of Embrapa Soja, Londrina, PR and the Research Station of the Farmers' Cooperatives initially performed production and formulation for the State of Paraná (COODETEC), Cascavel, PR. At that time, COODETEC hired two technicians to work at Embrapa Soja, in Londrina, to perform the quality control of formulated AgMNPV batches. In 1989, Embrapa started to negotiate contracts with private companies interested in producing and commercializing this biological insecticide. From 1990 on, five companies signed the contracts with Embrapa. Through these contracts, Embrapa would transfer all the technology for AgMNPV production in the laboratory and in soybean growers' fields, formulation and quality control of production batches. The companies would each pay Embrapa US\$40,000 initially, plus 5% of royalties of the AgMNPV sales each year, for a period of 10 years. The products based on the AgMNPV of each company were registered according to the Brazilian policies for registration of plant protection insecticides. All tests related to physical and chemical aspects of the formulations, toxicological data, identity (characterization), etc. were performed and approved by the Brazilian agencies involved (Agriculture, Health and Environment) (Moscardi and Sosa-Gómez, 1996). However, owing to the lack of experience with this type of product in these agencies, registration took about 3 years from the time of submission of all the required documents.

With the commercialization of the AgMNPV by private companies, use of the biological insecticide increased from about 1 million ha in 1990 to approximately 1.5 million ha in 1995, with most of the production being produced in the field during each soybean season. In addition to the early efforts by Embrapa to develop and improve technically and economically *in vivo* procedures for AgMNPV mass production under controlled laboratory conditions, two of the private companies also attempted to develop such production methodology. One of them (Geratec) was able to produce around 150,000 ha-equivalent of the virus per year in the early 1990s; however, owing to the high cost of labour, disposable rearing containers and components of the insect diet (mainly agar and casein), laboratory production of the virus was discontinued by the two companies. On the other hand, AgMNPV field production became widely adopted by all companies as the best available method to obtain large quantities of virus-killed larvae at a low cost.

AgMNPV Field Production

Field production of the virus became a big business, involving different persons and small companies that have specialized in selling AgMNPV-killed caterpillars

to the private companies that registered the product for commercialization (Moscardi, 1999). It involves impressive logistics. Growers' fields are contracted, and the pest control in their fields is implemented by the AgMNPV producers. Usually two to three fields are sprayed with the AgMNPV every day during the most prevalent *A. gemmatilis* larval occurrence (December and January). Before the collection phase, fields sprayed each day are inspected to select the one that will yield highest amounts of dead larvae. Peak collection occurs from the 8th to the 10th day in each selected field, and may involve from 200 to 300 'larval pickers'/day, requiring around 10 buses to transport them to the fields. In a single day, production in one collection site may reach 600 kg of AgMNPV-killed larvae, enough to process the virus for treatment of ca. 30,000 ha. Just to exemplify the importance of this production method for the AgMNPV programme, in the 2002/03 soybean season, approximately 45 t of AgMNPV-killed caterpillars were collected and sold to the private companies (about US\$ 10–12/kg), representing about 2.0 million ha-equivalents of the biological insecticide. This material is stored at -10°C for further processing and formulation (Moscardi and Sosa-Gómez 2000). Samples of each formulation batch are sent to Embrapa Soja for quantitative and qualitative (bioassays) quality control procedures before packaging.

Despite its value for producing high amounts of the AgMNPV at low cost, field production presented some problems that restricted the expansion of its use or affected the quality of the end product. Such problems include: (i) yearly production is dependent on the natural incidence of the host insect, which may occur in low numbers in certain seasons; (ii) production in the last 6 years has not been sufficient to meet demand. In fact, production has been only 20 to 30% of the demand. For example, production at COODETEC was approximately 700,000 ha-equivalent of the virus in the 2002/03 season, which was sold out by May before the next soybean season. However, this private company had an additional demand for about 300,000 ha, which could not be met; and (iii) owing to the pressure on virus field producers for AgMNPV-infected cadavers, quality of the field-collected material began to decrease as well as quality of the end product. The major problem is that collection of dead larvae in the field shifted from hand-picking to shaking plants over pieces of cloth placed over the ground in between soybean rows. This shift resulted in collection of dead larvae, live host larvae (with low amounts of virus), larvae from other lepidopterous species, other insects (stink bugs, beetles, etc.), and debris, resulting in material with higher amounts of extraneous organic matter other than the AgMNPV-killed larvae. While the hand-picking method resulted in an average of 50 ha-equivalent of the virus/kg, the current procedures resulted in an average of 30–35 ha-equivalent of the virus/kg. The higher amount of extraneous organic matter led to problems of nozzle clogging and efficiency of the biological product in the field. Therefore, commercial laboratory production of the AgMNPV became a must, in order to meet the increasing demand for the biological insecticide and improvement of its quality. To achieve this goal, research efforts were developed at Embrapa Soja to improve the commercial laboratory production methodology.

A Breakthrough in Commercial Laboratory Production

Improvements made in the AgMNPV laboratory production procedures up to 1997 (Abot, 1997; Moscardi *et al.*, 1997) served as a starting point for a 4-year PhD study (Santos, 2003) aimed at solving the most important bottlenecks related to commercial production of the AgMNPV. The main points worked out to increase production efficacy and to reduce costs were: (i) changing the agar for other gelifying agents and reducing the amount of casein, since these ingredients corresponded to over 90% of the insect diet cost; (ii) testing different rearing containers available in the local market to replace expensive disposable ones; and (iii) adjusting other production parameters, such as viral dosage, size of larvae at inoculation, number of larvae/container and temperature, for maximum virus yield.

Significant progress in AgMNPV production was attained as a result of this study. Diet cost was reduced by approximately 85% by substituting Carragen GP-911 for the agar and by reducing the casein content by 50%. With these new procedures, the cost of AgMNPV dead larvae to treat 1 ha was approximately US\$ 0.42, as compared with US\$ 0.29 for those collected in the field. Considering the much higher quality of laboratory-produced AgMNPV and the costs involved, results indicated that the final product obtained in the laboratory could be offered in the market at a lower cost than the chemical insecticides available to control the target insect. Therefore, a 'Pilot Laboratory' for AgMNPV production was proposed (Santos, 2003), in order to validate the results and adjust procedures at larger scale and transfer the new technology to private companies. This laboratory would have two independent sections: one for insect production and the other for virus production, involving the inoculation of 25,000 to 30,000 *A. gemmatilis* larvae/day, aiming at a yield of 2 t of dead larvae or about 100,000 ha-equivalent AgMNPV/year.

Although government monies were promised for implementation of a pilot laboratory at Embrapa Soybean in Londrina in 2002, in the end, the grant money was diverted to other government 'priorities'. Since this was an urgent matter, contacts were made with one of the private companies (COODETEC), which accepted implementation of the 'Pilot Laboratory' at its own risk. COODETEC rented a house in Cascavel, PR, the location of its headquarters, renovating it as a pilot production facility, with production commencing in May 2003. Results were so favourable that COODETEC decided to maintain the rented house for insect production but expand the AgMNPV production to two rooms at its headquarters. In this way, COODETEC were inoculating over 100,000 larvae/day by the end of 2003 and hiring 14 employees, obtaining a final AgMNPV product at a lower cost than the cost of chemical insecticide application. After processing over 1000 kg, yield was 65 to 72 ha-equivalent/kg of dead larvae. COODETEC then decided to build large laboratory facilities, inaugurated in November 2004. These consisted of two independent laboratories of 750 m² each: one for insect production and the other for virus production, with another existing facility (500 m²) being used for virus storage, processing and formulation.

In the first laboratory, eggs are obtained daily in adult oviposition rooms, and larvae are reared in separate rooms up to the fourth instar in 500 ml cardboard cups containing insect diet. Daily, 3% of the larvae are transferred to plastic trays with diet and vermiculite to obtain pupae and maintain the insect colony. The rest (97%) of the fourth-instar larvae are taken to the virus production laboratory, where they are transferred to plastic trays containing AgMNPV-treated diet. Seven days later dead larvae are collected into plastic bags with a modified hand vacuum cleaner. The larvae are then taken to a storage room for further processing and formulation of the product COOPERVIRUS PM. The commercial laboratory production started in December 2004, and when operating at full capacity, will be employing 45 people to produce and inoculate 800,000 to 1,000,000 larvae/day, resulting in AgMNPV to treat 1.8 to 2.0 million ha/year. Currently, this is the largest laboratory facility worldwide for the production of a viral insecticide, which is the result of many years of research and development.

With these achievements, Embrapa allocated funds to build a 120 m² laboratory for virus production (inoculation of 25,000 to 30,000 larvae/day), which was finalized in April 2005. The objective of this facility is to provide continuous improvements in the production process (e.g. reduce cannibalism, which is still high; test new rearing containers, etc.) and to provide training to other private companies interested in the commercial laboratory production of the AgMNPV. With these initiatives, a gradual substitution of laboratory-produced cadavers for field-collected ones is expected, generating enough product to meet the farmers' demands of a very high-quality biological insecticide.

Final Considerations

The use of the AgMNPV in Brazil has generated substantial economic, ecological and social benefits. At the soybean-grower level, considering that the cost of the AgMNPV is ca. 20% to 30% lower than the average cost of chemical insecticides and that the virus usually provides control during the season with only one application, compared with an average of two applications for chemical insecticides, the economic returns from the use of the virus may reach approximately US\$ 7.00/ha/season, which includes product and application costs (fuel, labour, etc.). Therefore the current annual savings at the soybean-grower level are over US\$14 million. Since implementation of the programme in the 1982/83 season up to the 2004/05 season, it is estimated that cumulative use of the AgMNPV reached approximately 23,000,000 ha, which represents savings to growers of about US\$161 million. Most importantly, since the beginning of the programme, more than 23 million litres of chemical insecticides were not sprayed in the environment, resulting in considerable benefits to society. Also, the programme has contributed to socio-economic improvement of poor families in AgMNPV field-production regions in the south, as hand-picking of virus-killed larvae during the soybean season has provided an additional and important income. Another important aspect is that the programme has contributed to changing

the profile of the insecticides used to control *A. gemmatalis* larvae in soybean (Moscardi *et al.*, 2002).

The programme for AgMNPV use in Brazil has been successful owing to various reasons: (i) implementation of a soybean IPM programme in the country, facilitating adoption of the AgMNPV by soybean growers; (ii) proactive activities of official extension services in transferring the AgMNPV technology; (iii) high virulence of the pathogen to the host and efficient horizontal transmission in the host population by biotic and abiotic factors, allowing control of *A. gemmatalis* with only one application during the season; (iv) continual exposure of the insect to the applied AgMNPV; (v) soybean tolerates high defoliation (30–40%) (high economic injury level) without significant yield reduction; (vi) usually, in most regions, there are no other simultaneous key pests, allowing use of a highly selective biological insecticide; and (vii) the ability for production of large quantities of the virus under field conditions at a very low cost.

With recent achievements in commercial production of the AgMNPV under controlled laboratory conditions, it is expected that the use of the biological insecticide may reach 4.0 million ha/year within 5 years. For the sustainability of the programme, research has been conducted regarding the possibility of *A. gemmatalis* developing resistance to the virus (Abot *et al.*, 1995, 1996), the possibility of viral attenuation after repeated mass production in the field or in the laboratory, as well as improving the AgMNPV formulation, among other aspects. The success of the programme also depended on long-term research projects, supported mainly by public funds (Embrapa), consisting of a stepwise approach towards the final goal (biological product available and used by soybean growers). At each of the steps, initiated 25 years ago, problems were solved with creativity and, mainly, perspiration, with those involved working every day, publishing less scientific papers than they should, in favour of participating in the technology transfer aspects. This sacrifice paid off for those involved, as the use of the AgMNPV at the farmer level became a reality and is internationally recognized as the largest programme of commercial use of a viral insecticide.

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39 **AbietivTM, a Viral Biopesticide for Control of the Balsam Fir Sawfly**

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Overview: The balsam fir sawfly is an indigenous pest of coniferous forests in North America. Since 1990, outbreak populations of balsam fir sawflies and subsequent defoliation of balsam fir have been especially severe in western Newfoundland. A species-specific nucleopolyhedrovirus has been known to cause balsam fir sawfly population declines in the past. This is the story of the isolation, development and registration of this virus for use in operational control programmes directed against this forest insect pest.

The Balsam Fir Sawfly

The balsam fir sawfly (BFS), *Neodiprion abietis* (Hymenoptera: Diprionidae), is an indigenous defoliator of spruce and fir across North America. In Newfoundland, Canada, it feeds primarily on balsam fir (*Abies balsamea*); populations reach epidemic levels for 2–4 years before collapsing. Population declines are associated with a nucleopolyhedrovirus (Baculoviridae), specifically NeabNPV. In late summer and early autumn, adults emerge from cocoons, and lay eggs individually into balsam fir needles produced that year. Eggs overwinter and hatch in late June to mid-July, and larvae feed on foliage that is 1 year old and older. Larvae spin cocoons in August. For 50 years prior to 1990, outbreak populations of BFS in Newfoundland lasted for only 2–4 years before collapsing, and impacted relatively small areas of balsam fir stands (Moreau, 2006). In 1990, when populations began to increase in Bottom Brook, south of Corner Brook, in western Newfoundland (Fig. 39.1), it was assumed that they would collapse within 5 years. However, this did not happen and the infestation spread, so that by 2006 a total of approximately 335,000 ha had been defoliated to some degree.

The forest industry is vital to western Newfoundland, and significant resources in terms of money and manpower have been invested in silvicultural practices such as precommercial thinning (PCT). Thinning, where the number of

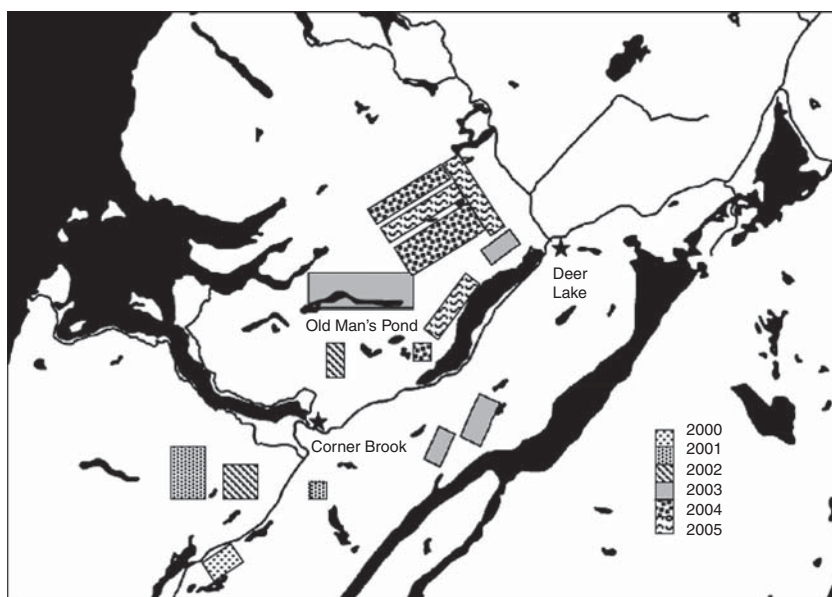


Fig. 39.1. Locations of NeabNPV (*Abietiv*) field trials 2000–2005 in western Newfoundland.

stems per hectare is reduced and the space between them increased by cutting down unwanted trees, is a common silvicultural technique practised worldwide. Balsam fir regenerates naturally, and thinning is typically carried out when trees are 3–5 m in height. Thinning affects tree and stand architecture, foliar chemistry of the remaining trees, illumination profiles, understorey composition, soil temperature, organic matter decomposition and mineralization. The effects of thinning on the vegetation may also affect the vertebrate and invertebrate inhabitants of treated forest stands. Thus, it was suspected that the increased duration and severity of the current BFS population outbreak was somehow connected to the extensive PCT tracts of balsam fir in western Newfoundland.

A Research Group is Formed

For this most recent outbreak, there were no commercial products registered for use against BFS, and ecological information on the BFS was limited. Further complicating the issue was a programme review within the Canadian Forest Service (CFS), beginning in 1994, which saw the downsizing and closure of the Newfoundland regional laboratory in St. John's and construction of a smaller facility in Corner Brook to be administered from Fredericton, New Brunswick as part of the newly created CFS–Atlantic Forestry Centre (AFC). Over the course of this process, one of the two CFS research entomologists in the Newfoundland region left the Service and the other accepted a new assignment at the CFS – Pacific Forestry Centre in Victoria, British Columbia. With these departures and because

the former Maritime and Newfoundland regional laboratories were merged into CFS–AFC, a group of scientists from Fredericton was assigned to tackle the BFS problem in Newfoundland. Initially, principal researchers included Mr Edward Kettela, Drs Eldon Eveleigh, Christopher Lucarotti, Don Ostaff, Harald Piene, Graham Thurston (all of CFS–AFC), Dan Quiring (University of New Brunswick), and David Levin (University of Victoria). In 1997, research funding was sought and received by the group through a Natural Sciences and Engineering Research Council of Canada (NSERC)–CFS–Industry programme, where the industrial partners included Corner Brook Pulp and Paper (Kruger), Abitibi-Consolidated, and the Newfoundland and Labrador Department of Natural Resources (NLDNR). Three years later, Levin, Lucarotti, Quiring and Dr Basil Arif (CFS – Great Lakes Forestry Centre, Sault Ste. Marie, ON) were among the founding members of the Biocontrol Network (funded by NSERC) (Fig. 39.2).

Balsam Fir Sawfly Impact

BFS larvae feed predominantly on foliage that is 1 year old and older, and the later instars are responsible for most of the defoliation. Generally, BFS do not kill affected balsam fir trees, but they can greatly reduce growth rates and make affected trees more susceptible to other defoliators. Removal of older foliage results in reduced size of current-year needles and a reduction in the number of needle primordia in developing buds. Loss of the older foliage, and the effect on current-year foliage, reduces the photosynthesizing biomass of affected trees, resulting in reduced incremental growth. Recovery of growth following severe defoliation can be slow, largely because only the older foliage is eaten. Thinning appears to increase the overall severity of BFS defoliation in PCT balsam fir stands.

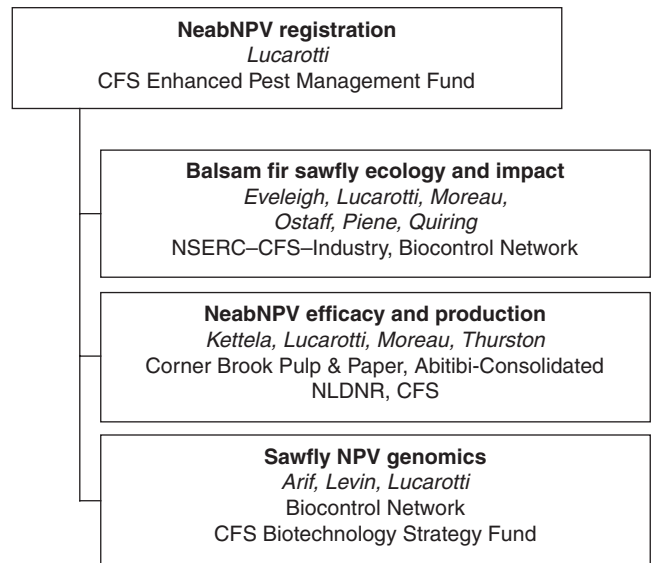


Fig. 39.2. Research priorities (bold), persons responsible (italics and alphabetical order), and sources of funding. Funds and/or in-kind support were also given by FPL, Fundy Model Forest, Ontario Ministry of Natural Resources, SERG International, and Société de protection contre les insectes et maladies (SOPFIM, Québec).

The increased defoliation occurs because population densities are greater in thinned stands. Higher population densities appear to be due to a reduction in larval mortality associated with natural predators, pathogens and parasites and the host plant in thinned stands.

The Virus

Baculoviridae is a family of viruses with covalently closed, double-stranded DNA genomes (Granados and Federici, 1986). Currently, there are two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). Baculoviruses are highly host specific and are restricted to insects, especially Lepidoptera and sawflies (Symphyta). Baculoviruses are transmitted through ingestion by a suitable host larva. In NPVs, virions are ingested as inclusions within protein (polyhedrin) occlusion bodies (OBs). Within the larval midgut, OBs dissolve, releasing virions to infect midgut epithelial cells. Lepidopteran NPVs go through an initial phase of replication in the midgut epithelium, producing virions that individually bud out of the midgut cells and spread the infection to the fat body and other tissues within the haemocoel, where further replication cycles take place in the nuclei of affected cells. In the late stages of replication, virions are enveloped and occluded into OBs. When lepidopteran larvae die from NPV infection, they often consist of little more than exoskeletons filled with OBs. Sawfly NPVs, however, only infect the midgut epithelium, so, following initial viral replicative cycles, infected midgut cells containing OBs rupture or are sloughed off into the frass and out of the body, where the virus can infect other host insects. Death normally occurs within 1–2 weeks, but during that time, the host is producing infective units of the disease. Host-specific NPVs have been used successfully against a number of sawfly forest pests, and NeabNPV was generally known to be the primary cause of the collapse of BFS populations in Newfoundland. Thus, NeabNPV was selected as the most likely candidate for successful use in control programmes against BFS.

Research Permits

One of our top research priorities was to carry out NeabNPV efficacy trials in the field. To do this, research permits had to be obtained, each year, from the Pest Management Regulatory Agency (PMRA) of Health Canada. This can take up to 180 days, but we have not had to wait longer than 140 days. The next step varies between provinces, but in Newfoundland and Labrador, a proposed efficacy trial must first undergo an environmental assessment review, which takes 45 days. Application for an environmental assessment can only be made with the PMRA research permit in hand. Thus, if an efficacy trial is to be carried out in mid-July, the application for a research permit has to be submitted to the PMRA by mid-December at the latest ($180 + 45 = 225$ days). In the locality of the trial, there will probably be additional requirements, such as public notifications

published in the local press usually 15–30 days in advance of the trials. Not being aware of these types of requirements could delay the trials for a year or more.

Virus Production Strategy

There are no *in vitro* cell culture systems for the production of sawfly NPVs. There are also no artificial diets on which sawfly larvae can be reared to adults successfully. Thus, sawflies must be reared on foliage of the host plant either on cuttings in the laboratory or on the plant in the field. Production of NeabNPV on BFS-infested balsam fir trees in the field was the only practical option to produce sufficient NeabNPV for aerial applications against BFS. We had originally isolated NeabNPV from BFS collected south of Corner Brook in 1997. Between January 1998 and March 1999, BFS larvae were reared in the laboratory on balsam fir foliage and were infected with NeabNPV. Sufficient NeabNPV was produced to attempt field production in July 1999. Our total laboratory production of NeabNPV (3.3×10^9 OBs) was applied, in 50 litres of 20% aqueous molasses, by helicopter on 2 ha of balsam fir forest. From this application, enough NeabNPV was obtained to treat 1800 ha of forest at an application rate of 1×10^9 OBs/ha. Subsequently, each year, we have produced NeabNPV in the field by making applications using fixed-wing aircraft over balsam fir stands (20–75 ha) heavily infested with BFS larvae (500–1950 eggs per 45-cm branch at the previous autumn egg count). Applications for the production of NeabNPV were made during mid-second to early third larval stadia. After 1 week, and for the next 2–3 weeks, trees were beaten with garden rakes and falling larvae were collected on tarpaulins placed under the trees. The contents of the tarpaulins were dumped into 40-kg sugar bags, balsam fir foliage was added, and the contents were treated with additional virus from a hand-held atomizer. The bags were clipped shut and placed in a large room, where the larvae could feed and die over the next couple of weeks. At the cessation of feeding, when larvae had either died or pupated, the added branches were removed and the contents of the sugar bags were transferred to and stored in 0.03-m^3 brown paper bags. Dead larvae were separated from the needles, rehydrated and homogenized. The virus was then semi-purified by filtration and centrifugation. NeabNPV OBs were quantified microscopically and brought to a concentration of 4×10^9 OBs/ml in water. As 2.5 litres of the 20% molasses solution are applied per ha, 1 ml of the virus concentrate is added to each 10 litres of the molasses solution to yield an application rate of 1×10^9 OBs/ha.

NeabNPV Field Efficacy

What made the efficacy trials possible was the fact that the maximum area that can be treated on a PMRA research permit in forestry is 5000 ha. Between 2000 and 2005, we were able to experimentally apply NeabNPV to approximately 22,500 ha of balsam fir forest. The cost of the efficacy trials was paid for, in large

part, by Corner Brook Pulp and Paper, Abitibi-Consolidated and NLDNR, and that cost worked out to less than \$80/ha treated, all included.

On 22–23 July 2000, three blocks, each 50 ha in area, between Pinchgut Lake and Big Gull Pond near Corner Brook, were treated aerially (Fig. 39.1). In all efficacy trials, NeabNPV was applied at a rate of $1\text{--}3 \times 10^9$ OBs/ha using Cessna 188 AgTruck airplanes equipped with Micronair AU4000 atomizers (Fig. 39.3). Aerial field trials were conducted on 21–22 July 2001 east and north of Stag Lake (2200 ha) near Corner Brook, and north of St. Alban's, Bay D'Espoir (600 ha) on 24 July 2001. On 21–23 July 2002, approximately 5000 ha were treated in three blocks to the south, east and north of Corner Brook. In 2003, 2004 and again in 2005, NeabNPV was applied to a number of blocks totalling approximately 5000 ha in each year. The locations of these application blocks were around Old Man's Pond, north of Deer Lake to Highway 430 and to the south-west of Pasadena. Analysis of the data from efficacy trials carried out in 2000–2002 (Moreau *et al.*, 2005) showed that, in the weeks that followed the treatment, both levels of NeabNPV infection and frass production increased in association with larval instar. However, levels of infection increased more rapidly in treated than in control blocks. In parallel, frass production was 31% lower in treated than in control blocks. Depending on the rate of change of populations, variable results with respect to insect density were observed in the year following the aerial spray. With increasing populations, as in 2000 (positive rate of change in control blocks), egg to third-instar density was almost one order of magnitude lower (tenfold difference in density) in treated than in control blocks in the year following the NeabNPV application. With peaking populations, as in 2001 (rate of change close to zero in the control block), egg to third-instar density was half an order of magnitude lower in the treated than in the control block



Fig. 39.3. An FPL Cessna 188 sprays Abietiv over a Newfoundland forest.

in the following year. With decreasing populations, as in 2002 (negative rate of change in control blocks), egg to third-instar density was similar in treated and control blocks. The results obtained in 2000–2002 suggest that increasing or peaking population outbreaks of BFS can be successfully suppressed by aerial applications of NeabNPV at rates as low as 1×10^9 OBs/ha.

Registration

Current requirements to register microbial pesticides in Canada are detailed by the PMRA in the regulatory directive, DIR2001-02: Guidelines for the Registration of Microbial Pest Control Agents and Products (this and additional information can be obtained from: www.pmra-arla.gc.ca). As one would expect, the various non-target toxicological tests, environmental impact studies, efficacy trials, and the like are all listed in this document. Officials from the PMRA should be consulted as early as possible in the research programme to develop a microbial control agent, and once again before submitting the registration documentation. During these 'pre-submission' consultations, scientists and regulators from PMRA will provide the prospective applicant with a DACO (data-code) table listing what tests are required (i.e. Table 39.1), conditionally required, where scientific literature may suffice, and so on. It was our experience that these consultations save a lot of time and money. Submission of the data and documentation in support of product registration must conform to an exact format. Here, we strongly advise that a consultant with extensive experience with the requirements of the PMRA for these submissions be contracted. This too, in the long run, will save much time, money and a great deal of frustration.

Commercialization: Abietiv is Born

We have registered the trade name 'Abietiv' for our NeabNPV product. The name derives from the specific epithet of *N. abietis* and the 'v' is for virus. In May 2005, CFS–AFC signed a licensing agreement with Forest Protection Limited (FPL), a private company located in Fredericton whose board of directors includes representatives from the provincial government and the forest industries of New Brunswick. FPL is responsible for fire protection and pesticide application in the forests of New Brunswick. It also has research interests in aerial application technologies and pesticide development. FPL and CFS–AFC have long had a cooperative approach to forest protection research in New Brunswick and elsewhere. FPL was the obvious choice for the commercialization of Abietiv. FPL, in cooperation with BioAtlantech, has established a company, Sylvar Technologies Inc., to market Abietiv, and develop and commercialize other baculoviruses.

The Bottom Line

Factoring in salaries, use of facilities, equipment and vehicles, contracts, efficacy trials and the rest, we estimate that the total cost from isolation of NeabNPV to

Table 39.1. List of invertebrate and vertebrate test animals exposed to NeabNPV and associated effects of that exposure.

Non-target taxon	NeabNPV Dose	Toxicology
Hymenoptera		
<i>Acantholyda erythrocephala</i> (Pamphiliidae)	1 × 10 ⁶ OBs/larva	Significant mortality ^a
<i>Diprion similis</i> (Diprionidae)	1 × 10 ⁶ OBs/larva	Significant mortality ^a
<i>Gilpinia hercyniae</i> (Diprionidae)	1 × 10 ⁶ OBs/larva	Significant mortality ^a
<i>Pristiphora geniculata</i> (Tenthredinidae)	1 × 10 ⁶ OBs/larva	Significant mortality ^a
<i>Apis mellifera</i> (Apidae)	1 × 10 ⁷ OBs to hive	None
<i>Megachile rotundata</i> (Megachilidae)	1 × 10 ⁶ OBs/larva	None
Lepidoptera		
<i>Clepsis persicana</i> (Tortricidae)	1 × 10 ⁶ OBs/larva	None
<i>Choristoneura rosaceana</i> (Tortricidae)	1 × 10 ⁶ OBs/larva	None
<i>Melanchra pulverulenta</i> (Noctuidae)	1 × 10 ⁶ OBs/larva	None
<i>Pyrrhia exprimens</i> (Noctuidae)	1 × 10 ⁶ OBs/larva	None
Crustacea: Decapoda		
<i>Daphnia magna</i>	1 × 10 ² –10 ⁶ OBs/ml	None
Vertebrata: Mammalia		
Sprague-Dawley rats Oral gavage	Single dose 1 × 10 ⁸ OBs	None
Sprague-Dawley rats Intravenous injection	Single dose 1 × 10 ⁷ OBs	None
Sprague-Dawley rats Intratracheal instillation	Single dose 1 × 10 ⁸ OBs	None
CD1 mice Intraperitoneal injection	Single dose 1 × 10 ⁷ OBs	None
New Zealand white rabbits Topical application to bare skin	Single dose 2 g /kg body weight	None

^aDot-blot hybridizations using NeabNPV DNA were negative, indicating little or no NeabNPV replication.

registration of Abietiv has been around CA\$6 million. The specificity of NeabNPV and the lack of potential for cost recovery, not to mention profit, were deterrents to investment from the pesticide industry so most of the cost has been borne by the CFS. Funding from NSERC contributed greatly to the basic research, directly by funding graduate students and their research, and indirectly in that

NSERC funds, along with additional funds obtained from CFS (Fig. 39.2), helped leverage monies from other sources. Because of the threat posed by BFS to the economy of western Newfoundland, the provincial government and the local forest industries (the end-users of Abietiv) invested in the development of Abietiv as a biological control agent for the BFS by largely covering the direct costs of the efficacy trials.

The success of the BFS project rests with the collaborative efforts of many people, including scientists, technicians, students, field and laboratory assistants, administrators, regulators, contractors and others. It also involved the cooperation of universities, agencies of the federal and provincial governments, and members of the forest industries in Canada. In a large, multifaceted project such as the one described here, it is important that at least one person be charged with the duty of seeing that the main objective is achieved. In our case, that job was the registration of Abietiv and it fell to one of us (C.J.L.) (Fig. 39.2). In no way did he direct the research programme overall or the research of individuals other than those reporting directly to him. His primary function in this effort was to ensure that all the relevant data and information from the various research components of the project made it into the Abietiv registration package submitted to PMRA, and that the package passed through the registration process successfully. (An electronic version of the Abietiv registration dossier is available in Lucarotti *et al.* (2006).)

Abietiv was registered as a biological control product for use against BFS in April 2006. In July 2006, NLDNR took delivery of a supply of Abietiv from Sylvar Technologies and applied it aerially over 15,000 ha of balsam fir sawfly-infested forest in western Newfoundland.

Acknowledgements

Research support was provided by those agencies listed in Fig. 39.2 and is gratefully acknowledged. Thanks are also given to the many people who assisted in this endeavour.

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40

Field Tests in the UK of a Genetically Modified Baculovirus

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Overview: Foreign genes expressing insecticidal proteins have been introduced into baculoviruses to improve their efficacy. These recombinant baculoviruses were shown to paralyse and kill target larvae faster than the wild types. In order to find out whether genetically modified baculoviruses had any potential as effective and safe pest control agents, these viruses had to be field tested under very strict regulatory constraints. This chapter describes a series of field and laboratory tests initiated soon after the first genetically modified baculoviruses were produced.

Introduction

As with most microbial pest control agents, wide-scale development and uptake of insect baculoviruses has been limited by a number of factors, one of which is their comparatively slow speed of kill. In part, this perceived need relates to the use of most microbial control agents as part of inundative short-term control programmes, rather than utilizing their capacity to recycle and be augmented within longer-term control scenarios. Lepidopteran baculoviruses possess several interesting features, the most obvious of which is that their virions (or virus particles) are occluded in a protein coat. It is this protective coating that makes them stable enough to be applied in spray applications for insect pest control. For the two morphological baculovirus types, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs), the protein that makes up the coat, polyhedrin and granulin respectively, is expressed at very high levels. This feature, and the fact that it is not essential for baculovirus replication, attracted the attention of molecular biologists looking for novel ways of producing proteins. This led to the development of the first baculovirus expression vectors, in which the polyhedrin gene was replaced by a foreign gene (Pennock *et al.*, 1984; Smith *et al.*, 1985). Since these early developments, baculovirus expression vectors have been used to produce a multitude of proteins, either singly or in combinations, and under the control of a variety of different promoters.

The development of the baculovirus expression vector also provided the means by which the baculovirus could potentially be 'improved', as the same technology could be used to express genes that might alter the biology of the virus. The first aim of the genetic modification of baculoviruses was to try and increase the speed with which they killed the target pest or reduced their feeding. Various approaches were adopted, including the expression of insect hormones and enzymes; however, the first genes that were inserted into baculoviruses that successfully altered their biology were insect-selective toxin genes derived from other arthropods, namely the North African scorpion, *Androctonus australis*, and the predatory mite, *Pyemotes tritici* (McCutchen *et al.*, 1991, Stewart *et al.*, 1991, Tomalski and Miller, 1991). In laboratory assays, these genetically modified viruses were shown to paralyse insect larvae, resulting in a more rapid cessation in feeding and earlier death. This breakthrough led to the construction of baculoviruses expressing a range of different genes. However, while the construction and testing of genetically modified baculoviruses in the laboratory could progress at a relatively rapid rate, the real test was whether these recombinant viruses could make a difference in the more variable crop environment. In order to find out whether genetically modified baculoviruses had any potential as effective and safe pest control agents, we initiated a series of field and laboratory tests soon after the first genetically modified baculoviruses were produced.

Efficacy Testing of a Recombinant Baculovirus in the Field: the First Steps

In 1993 we carried out the first field test of a genetically improved baculovirus. All of the early genetic modification of baculoviruses was carried out on NPVs, primarily *Autographa californica* MNPV (AcMNPV), as these had better-developed cell culture systems and a greater wealth of molecular data, making them more straightforward to modify. Thus the options for a field release were limited. For our field studies we chose an AcMNPV clone modified to express a toxin, AaIT, from the scorpion, *A. australis* (Stewart *et al.*, 1991). Part of the rationale behind this choice was that this toxin had been well characterized in terms of its toxicity, and had been shown to be insect selective in its action, which therefore reduced the breadth of the testing needed prior to its release. We opted to carry out a contained field trial so we could control the fate of the virus and its target host. The target was the cabbage looper, *Trichoplusia ni*, a species that is highly susceptible to AcMNPV. Before being able to release a recombinant baculovirus into the field it was necessary to obtain permission from the Advisory Committee for Releases into the Environment (ACRE), in addition to various other bodies such as the UK Department of the Environment and the then Ministry of Agriculture, Fisheries and Food. Application to ACRE involved completing a detailed risk assessment relating to all aspects of the organisms to be released: their molecular make-up, biology, target environment, potential hazards and methods for mitigation. This was a non-trivial exercise, involving many probing follow-up questions, discussion and finally an appearance before the committee in London.

As a result of the extended discussion period and the resulting delay in permission being granted, it was difficult to plan the trial itself as it required several weeks notice for growing the crop plant. Permission was eventually granted at the beginning of August 1993 and the trial went ahead in early September.

Aims of the Field Testing Were to Verify Laboratory-acquired Information on Speed of Kill and Host Feeding Behaviour

The aim of the field trial was primarily to see whether the differences in speed of kill and feeding behaviour seen in the laboratory actually resulted in measurable differences in crop protection in the field, as the more variable field conditions will often override what appear to be significant differences in carefully controlled laboratory experiments. However, I also wanted to show the mechanisms behind any differences in crop protection, i.e. that they resulted from the differences between the two viruses produced by the expression of the scorpion toxin gene (or some other cause). The laboratory bioassays had shown that the AcMNPV recombinant expressing AaIT reduces time to death by approximately 25%. Thus I wanted to design a sampling regime that made it possible to confirm that insects infected with the AcMNPV-AaIT recombinant were paralysed before insects were killed by the wild-type virus, in addition to examining whether feeding had actually been reduced. As tends to be the case, the laboratory bioassays had been carried out at a constant high temperature and thus it was hard to extrapolate to the lower and more variable field conditions, which would invariably extend the infection period. Based on experience with using NPVs as control agents in other systems, I eventually plumped for a four-time-point sampling regime that covered the likely infection period but allowed for some flexibility if the field temperatures suddenly changed.

Treatment with a Recombinant Baculovirus Significantly Reduced Crop Damage Compared with an Unmodified Virus

Details of the field trial set-up and results can be found elsewhere (Cory *et al.*, 1994). However, in brief, cabbage seedlings were planted in 1 m³ netted enclosures (multiple enclosures per treatment), left to grow, and third-instar *T. ni* larvae were introduced 4 weeks later. The recombinant and wild-type viruses were compared (with untreated controls) as spray applications at three rates. Because of space constraints, each enclosure was sub-sampled at the four time-points (2, 7, 11 and 16 days after spraying) and at each time-point five cabbages were destructively sampled and all caterpillars on them reared through individually in the laboratory to assess the level of virus infection. At the end of the 16-day trial, leaf areas were measured to estimate crop damage. The leaf area analysis

clearly showed that not only did the virus-treated plots sustain less damage than the controls, but that the recombinant treatment protected the crop to a greater degree than the wild-type virus and this was concentration dependent. In terms of why this happened, analysis of the number of live larvae collected from each plot showed that insects collected from the recombinant treatments declined before those from the wild-type treatment, confirming the more rapid action of this virus. In addition, it was evident from visual inspection of the field site, and from both collection and survival data, that the larvae infected with the scorpion toxin virus fell off the plants when they became paralysed, reducing the possibility of further cycles of infection. Thus the field trial demonstrated that treatment with a recombinant baculovirus could significantly reduce crop damage compared with an unmodified virus. It also confirmed that these differences were the result of the recombinant virus acting more quickly. In addition, the trial demonstrated the importance of carrying out realistic tests of these viruses; laboratory assays would not reveal such crucial behavioural differences.

Ecological Risk Assessment of Recombinant Baculoviruses

The efficacy trial completed the first phase of testing of the recombinant baculovirus and demonstrated that even a relatively modest increase in speed of action could produce an increase in crop protection. The second, longer phase of the field trials moved on to assessing ecological risks that might be attached to the release of genetically modified baculoviruses. Baculoviruses possess various features that enhance their capacity to act as biological insecticides. For example, their protein coats endow them with the capacity to persist outside of their hosts and survive between generations and from one year to the next. In addition, the coat, and the alkaline conditions required to break it down, allows them to pass intact through the guts of a variety of predatory and scavenging organisms, such as beetles and birds, which can passively vector the virus over quite large distances. This aids the spread of the virus after it has been applied in the field. However, the same features that are seen as positive for successful pest control are also perceived as negative features when it comes to the ability to contain a recombinant virus after release. Thus various approaches to risk assessment have tended to highlight such 'negative' characteristics, and some approaches to genetic modification have also looked at decreasing features such as persistent capacity in order to make the viruses more environmentally safe (e.g. Hamblin *et al.*, 1990; Woods *et al.*, 1994). Our approach was a little different; we accepted that it was unlikely that a baculovirus could be contained after release, but focused on a more generic approach to risk assessment, where the impact of incorporating a foreign gene could be assessed against a background of the parent wild-type virus. We could thus make predictions about whether a novel baculovirus recombinant was fitter than its parent virus, and thus likely to replace it in a natural ecosystem (Cory, 2000).

The Main Perceived Hazards Resulting from the Release of Genetically Enhanced Baculoviruses: Disruption of the Pathogen Complex and Impact on Non-target Organisms

The main hazards resulting from the release of genetically enhanced baculovirus insecticides were seen as: (i) environmental perturbation resulting from disruption of the 'normal' pathogen complex by a novel recombinant with superior fitness; and (ii) negative impact on susceptible non-target species. Therefore, the first aim of the risk assessment studies was to assess how the incorporation of the scorpion toxin gene affected fitness. The second aim was to analyse how natural and recombinant baculoviruses affected non-target hosts. Non-target impact is perhaps the main risk from the release of any biological control agent, and for pathogens in particular it is an area that needs close scrutiny (Cory and Myers, 2000). Baculovirus host range is restricted and their risk to non-target species is limited to the order from which they were isolated, so viruses isolated from the Lepidoptera are only likely to kill other Lepidoptera. Even within the lepidopteran baculoviruses, many isolates have very restricted host ranges, often limited to single species, and so any non-target risk from these is likely to be negligible. However, AcMNPV has a larger host range than many baculoviruses and thus non-target impact was a key component of the studies. Part of our programme did include wide-ranging host range studies. Most research on host range tends to focus on the impact of the pathogens on other species which are pests, whereas testing for potential ecological risks needs to look beyond this. Much host range testing tends to be arranged phylogenetically, under the assumption that more closely related species are likely to be more susceptible. Our studies took a broader view, and while many species tested came from the noctuid family to which *A. californica* belongs, species from as many families as possible were tested. These assays indicated that host range for AcMNPV was not predictable and that, while closely related species could be highly susceptible, many closely related species were not, whereas some more distant species were susceptible to the virus at relatively low doses, and it was clear that many of the species tested were intermediate in their susceptibility (Cory, 2003). From these results we concluded that for relevant studies on non-targets, it was important to assess the response of species that showed a range of susceptibilities.

Does the Expression of a Toxin Gene Produce a More Competitive Virus?

Our approach to seeing if the expression of a toxin gene could produce a more competitive virus was to compare a range of different parameters for the two viruses. Specific features of the viruses (or any pathogen) will define its dynamics, for example the amount of virus that each caterpillar produces when it dies and the rate at which it is transmitted between hosts. Fitness is often defined as the basic reproductive ratio, R_0 , and is used to describe the ability of an organism to produce progeny that survive to contribute to the following generation. For pathogens, this tends to be modified to the number of hosts infected by one infected host (or

infectious unit) and thus is a combination of the productivity of an infection combined with the likelihood of infection being acquired by another host. If an organism has an R_0 of less than 1 it will fail to produce enough of itself to survive, whereas those with R_0 s greater than 1 will spread. While an absolute measure of R_0 is going to vary with conditions, it is possible to use this approach to assess whether the addition of a foreign gene, such as a toxin, will act to increase or decrease the basic reproductive ratio and thus whether a novel recombinant is likely to be fitter than its parent (see also Dushoff and Dwyer (2001) and Bonsall *et al.* (2005) for a mathematical analysis of competition between genetically modified baculoviruses with faster speeds of kill).

Laboratory Bioassays to Estimate Speed of Kill, Productivity, Transmission and Persistence

Estimates of speed of kill and yield could be gathered from laboratory bioassays (although they would undoubtedly be modulated by field conditions); however, parameters such as transmission and persistence are best estimated in the field as they rely on natural insect behaviour and environmental conditions. We started with a laboratory comparison of the AcMNPV parent C6 clone and the AaIT recombinant, comparing two host species: the highly susceptible *T. ni* and the cabbage moth, *Mamestra brassicae*, a species of intermediate susceptibility. These assays showed, as expected, that *M. brassicae* required approximately 58 to 150 times more virus for an LD₅₀ dose than *T. ni*, and that the two viruses were equally pathogenic for *M. brassicae* (Hernández-Crespo *et al.*, 2001). However, they also indicated that the recombinant virus was more pathogenic (requiring half as much virus for an LD₅₀ dose) than the wild-type in *T. ni*, a result that had not been found in any previous assays by ourselves or other groups using a similar virus construct. In terms of the parameters we wanted to estimate, we found that both yield and time to death were greater in *M. brassicae* than in *T. ni*. However, we also demonstrated that the recombinant did not kill *M. brassicae* more rapidly than the wild-type AcMNPV, although it still resulted in a reduced yield of occlusion bodies (OBs) (Hernández-Crespo *et al.*, 2001). This provided the first indications that it may not be possible to extrapolate from one species in terms of being able to predict the response of less susceptible non-target species to recombinant baculovirus insecticides.

Field Trials to Verify Laboratory-acquired Information on Speed of Kill, Productivity, Transmission and Persistence: Overcoming Negative Public Opinion

We applied to ACRE for a new permit for a field release, this time covering 5 years of trials, all geared towards addressing questions relating to possible ecological risks of releasing recombinant baculoviruses. As the application related to the same recombinant virus as before, which was being released at the same field

location and using the same containment and decontamination procedures as before (which had been shown to work well as recovery of insects from the enclosures was high), the process was completed relatively rapidly. The new series of field trials was approved in the summer of 1994. In spite of the success, both scientifically and environmentally, of the first trial, this time, however, the field trials attracted more public and media interest, in part due to the activities of certain local individuals. We had always adopted the approach of being open with the media, allowing them access to both the laboratory and the field site, and, in addition, had held open days where any interested parties could come and talk to the scientists. Also, prior to initiating the work we informed and discussed the programme with local conservation bodies, such as the Royal Society for the Protection of Birds, English Nature, naturalists' trusts and butterfly conservation organizations. However, in spite of these efforts, there was a small, but vociferous, local reaction to the trials that was not wholly positive, and some of this was picked up by the national media. We attempted to address and respond to the issues raised by each individual or organization, and as a result of this we produced a detailed fact sheet, which we sent to all those who contacted us. While some individuals remained to be convinced, we often found that many of our correspondents reacted very positively to the information received. It was apparent that their opposition was in part based on a lack of information (or sometimes misinformation), in particular relating to the nature and purpose of the trials, the biology of baculoviruses and their narrow host range, their commonness in nature and their history of safe use as bioinsecticides. In fact several correspondents wrote back to me, after receiving the fact sheet, to wish us good luck in the work!

Are Non-target Species at Risk?

Eventually, what was a very stressful time receded and we proceeded with the trials. My plan was to carry out a two-stage experiment that mimicked what happened in a field control situation. So the first trial would be a spray application on two species which varied in susceptibility, representing a target and a non-target species (*T. ni* and *M. brassicae* as in the laboratory experiment) and then this would be followed by a separate trial in which secondary transmission from cadavers killed by the viruses would be followed, i.e. mimicking natural transmission. The first trial was carried out in a similar manner to the 1993 efficacy trial, i.e. spraying virus on to caterpillars within 1 m³ contained plots, which were then sub-sampled at intervals and the larvae reared through in the laboratory. The only difference was in the timing of the field collections, which were collected at 1, 3 and 5 days post-spraying, to allow the larvae time to acquire the virus but not leave enough time for a new wave of virus-induced death to occur. The field trial supported most of the findings made in the laboratory bioassays: (i) the risk of infection for the less susceptible species, *M. brassicae*, was lower than for *T. ni*; (ii) both viruses were equally pathogenic for *M. brassicae*; (iii) *M. brassicae* larvae died later; and (iv) the recombinant virus killed *T. ni* larvae faster than the wild type (Hernández-Crespo *et al.*, 1999). However, the more controversial results

from the laboratory study were not supported; thus the viruses produced equal mortality in *T. ni* larvae (rather than the recombinant being more pathogenic) and the recombinant did not kill *M. brassicae* later than the wild-type virus and did in fact kill the insects collected from the last two time-points earlier. This latter difference is thought to be a feature of the different temperatures in the laboratory and the field, but both issues do illustrate that significant differences found in the laboratory can be negated by more variable conditions in the field.

Does Secondary Transmission from Cadavers Killed by the Viruses Occur?

The second field trial addressed the longer-term issue of virus transmission; the means by which either the wild-type or recombinant virus could cycle and persist in natural populations. The approach adopted was based on the work of Greg Dwyer (1991) and used a technique whereby the transmission parameter could be estimated from a short-term experiment that took place within a season. Dwyer's work was based on research with the gypsy moth and its NPV (Dwyer and Elkinton, 1993), and this was adapted for agricultural insects, initially working out a suitable experimental design using *M. brassicae* and its NPV (Goulson *et al.*, 1995). Transmission combines a measure of the innate susceptibility of the host with the likelihood of acquiring infection; thus for a pathogen that is ingested this means that allowing the host to behave naturally is important. We therefore used the same type of cages as in the previous trials, as this would allow the larvae to move freely over the cabbage plants. The trial was aimed at answering three questions: (i) how do measures of susceptibility attained in laboratory bioassays translate into infection levels in the field; (ii) how does expression of the toxin gene affect transmission; and (iii) how does transmission compare within and between hosts that vary in susceptibility? We addressed these issues by having eight treatment combinations; each of the viruses (AcMNPV and AcMNPV plus AaIT) was released in the cadavers of one of two species, *T. ni* and *M. brassicae*, and transmission could be to either of these two species. Our previous work allowed us to make several predictions: first, we expected transmission to *M. brassicae* larvae to be less than for *T. ni* as it was less susceptible; secondly, transmission of the recombinant virus should be reduced in the field as the paralysed insects fall from the plants; and thirdly, transmission from infected *M. brassicae* larvae should be greater than from *T. ni* larvae as the cadavers contain a greater OB yield. The trial was carried out in a similar manner to the previous trials, except that virus was released in the form of pre-infected insects and sampling was carried out on a whole plot basis rather than sub-sampling. The infected insects were left on the cabbages for a week before the healthy susceptible insects were released, to ensure that the donor insects had died. A series of models was then fitted to determine the probability of infection during the experiment. A separate persistence experiment was carried out to ascertain whether the amount of virus available changed during the course of the trial, which went on for 7 days (it did not).

Virus infection was acquired very rapidly after release of the susceptible insects, within the first 24 h, and then levelled off. This resulted in the rate of acquisition of infection (transmission) being the same for all treatments. An additional parameter was then incorporated into the statistical model to account for the apparent 'refuge' from infection, which provided a much better fit to the data (Hails *et al.*, 2002). The refuge in this case means that a significant proportion of the population escaped infection, either through physiological resistance or, more likely, by never encountering a virus-infected cadaver. To a large extent the results of the trial conformed to our expectations: the risk of infection was lower for the less susceptible species and infection levels were significantly reduced for the recombinant virus as a result of the insects falling off the plants when they became paralysed. However, the yield within the cadavers appeared to make no difference to the resulting levels of infection, at least within the range utilized in the experiment, indicating that the number of patches of virus present was more important for secondary transmission than the quantity of OBs that they contained.

This Series of Trials Formed the Core of the Field Releases in the UK

This series of trials was followed by two further experiments that investigated whether insect life history influences the risk of infection (soil versus foliar feeders) and one in which the dynamics of virus movement were looked at in more detail, neither of which will be discussed here. The results from the laboratory and field experiments clearly showed that expression of the scorpion toxin gene changes several facets of the biology of the virus that are likely to alter dynamics in the field. In particular, the reduced yield and levels of transmission of the recombinant are likely to reduce its fitness and the likelihood for persisting within natural insect populations; it should be out-competed by the wild-type virus. However, the field trials did not address one key parameter that could alter the outcome, environmental persistence. The last trial showed that virus availability (a combination of the rate of virus degradation, the dilution effect of plant growth and increased virus coverage through breakdown of the cadaver) remained constant during a week-long experiment. However, longer-term studies of persistence between generations were not possible owing to the contained nature of the trial and the limitations of the release. Changes in environmental persistence could have profound effects on virus–host dynamics, and it is difficult to judge from the current data as to how this will be affected by genetic modification. In the example of the AcMNPV expressing AaIT, the fact that the paralysed larvae tend not to lyse is likely to increase their longer-term environmental persistence, as is the fact that they are deposited on the soil, where they could be protected from UV degradation. However, we also need to know whether the virus in the soil is available to susceptible species or can be reintroduced. These uncertainties can only be addressed by further field trials.

Lessons Learned for Future Field Evaluations and Risk Assessments

Many different recombinant baculoviruses have since been produced, some utilizing toxins, several of which have a greater impact on speed of action than AaIT; others have taken different approaches, such as the expression of hormones and enzymes and utilizing the genes of the host insect (Feng *et al.*, 2001; Inceoglu *et al.*, 2001). A similar approach to testing and risk assessment can be adopted for any genetically modified pathogen. With improvement in cell culture and other techniques, many other baculovirus isolates can also now be genetically altered. Most of these have a narrower host range than AcMNPV, and several appear to be species specific, which greatly reduces any potential risks that may be attached to their environmental release. Several lessons can be learned from the experiences of the field releases in the UK. From the perspective of public attitude, the perceived risks of GMO release are seen as high, and perhaps more importantly, beyond individual control. Thus it is important to start information, education and discussion processes as early as possible and with as wide a range of groups as possible. From the scientific and practical perspective, the UK trials clearly demonstrated that it is not possible to alter a single trait without influencing other aspects of an organism's biology, and that these may have significant impacts on their efficacy or their ecology and evolution. In addition, it also showed the importance of carrying out trials under realistic field conditions, rather than relying solely on laboratory-generated data. Baculoviruses are extremely diverse, widespread and represent a common component of the lepidopteran ecosystem. In addition, Lepidoptera are infected by a wide range of pathogens, and in a more realistic scenario, any baculovirus is going to have to compete with a diverse background of pathogens and parasites. While there is no evidence so far to indicate that genetically modified (or natural) baculoviruses present an ecological risk, more investment is needed in meaningful, field-based risk assessment studies. This would include analysis of pre-release field conditions, in addition to monitoring post-release changes. The studies described relate to one system and only address some of the issues; other key aspects including environmental persistence and interaction with indigenous viruses and other microorganisms remain to be addressed.

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41 Control of Mites in Pome Fruit by Inoculation and Conservation

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Overview: In North America consumers are demanding high-quality fresh apples virtually free of any pesticide residues. Meanwhile, in addition to diseases of apples, there are over a hundred different species of arthropods that are direct or indirect pests of apple. Because of the extensive scientific information made available in recent years in a subject that can easily become overwhelming, we have focused our attention on biological control of phytophagous mites in Quebec, where we have been the principal researchers in recent years. We hope that this case history will generate enough interest for you to develop biological control programmes in other crop systems.

History

Biological control of phytophagous mites in Quebec orchards may be placed in two time periods. The first period was from 1979 to 1990 and was based on the rearing and inundative releases of *Neoseilus fallacis* (Phytoseiidae). It proved to be impractical on a large scale. The second period was from 1990 to date, and it was the development of a more robust and grower-friendly programme. This approach was based on the philosophy of conservation, augmentation and transfer of natural predators. It was based on a comprehensive understanding of the toxicity of pesticides used to manage other pests and diseases in orchards. The predators were *N. fallacis*, *Typhlodromus caudiglans* (both Phytoseiidae), *Agistemus fleschneri* (Stigmaeidae), *Balaustium* sp. (Erythraeidae), *Anystis baccarum* (Anystidae) and *Hyaliodes vitripennis* (Miridae). Winter- and summer-pruned wood from an orchard where biological control has been established was used to accelerate the recolonization of orchards where biological control was in the process of being established.

The 1960s: the era of laissez-faire and laissez-passer

This was the era of laissez-faire and laissez-passer. Prophylactic treatments based on the phenology of the apple tree were the order of the day. It was brought about by the discovery of the insecticidal properties of DDT and the organophosphates during World War II and the carbamates in the 1950s. An exception to this approach was the 'modified' spray programme developed in Nova Scotia (Pickett *et al.*, 1946) and adapted for Quebec orchards in the early 1950s (LeRoux, 1960). In the USA, *Silent Spring* was published in 1963 and DeBach published his *Biological Control of Insect Pests and Weeds* in 1964. However, these studies were taken lightly as treatments were inexpensive, and spray and count to verify the effect on the target pests had become institutionalized across North America. Meanwhile, the indiscriminate use of pesticides induced insurmountable mite problems, because of the accelerated development of resistant strains among pest species and the almost total destruction of predator and parasitoid populations in orchards.

The 1970s: the era of la lutte raisonnée (supervised chemical control)

The discovery of resistant strains of predacious mites by Downing and Arrand (1968) and Hoyt (1969) summoned a new age in biological control of mites in orchards. This was swiftly followed by the mass rearing, transport and release of predacious mites in regions that previously had only insecticide-susceptible strains of predators (Croft and Barnes, 1972). Meanwhile, in Quebec, a nominal action threshold of five motile forms of tetranychids was established, and acaricide treatments were rationalized to a pre-bloom treatment followed by mid-summer treatment.

The First Period

The 1980s: the era of biological control by inoculation

The late 1970s and early 1980s witnessed the birth of biological control of pest mites in Quebec apple orchards modelled after the studies of Wearing *et al.* (1978) in New Zealand, and Croft and Hoyt (1983) in Michigan, USA. The project was to be executed in four studies. The first study involved screening for naturally selected OP resistance, mass rearing, maintenance of OP resistance and effects of other pesticides used in orchards on the selected strain of predators. The second study established the most appropriate time for release and the number of predators that would be required for inoculative releases to achieve biological control of phytophagous mites. The third study was the integration of the techniques and know-how gained in the previous studies in an experimental block in an orchard. The fourth and last study was to scale-up the rearing of predators and to evaluate the programme in a commercial context.

The first study

A naturally selected OP-resistant *N. fallacis* (Phytoseiidae) (NF) from Dunham, Quebec, and from Vineland, Ontario, were crossed (Fig. 41.1). The progeny was then reared in greenhouses according to Rock and Yeargan (1970). The progeny showed no mortality or behavioural changes following treatments with 2.8 kg/ha of azinphos-methyl (Guthion® 50WP). The resistance was maintained by bi-weekly treatments of the mass-reared predators with phosmet (Imidan® 50WP) as azinphos-methyl posed a health hazard to greenhouse personnel. The mass-reared NF were also used to elucidate the effects of several insecticides and fungicides on this predator (Bostanian *et al.*, 1984, 1985).

The second study

Small-plot trials showed that the most appropriate time to inoculate a plot was when the European red mite (ERM), *Panonychus ulmi* (Tetranychidae), density was not over 2.5 mobile forms per leaf in spring. Below that threshold, variations in ERM numbers within the block made ERM numbers statistically inaccurate. Furthermore, there would not be enough prey per leaf to sustain a predator release. On the other hand, above 2.5 ERM per leaf, a predator-prey ratio in favour of the predator was difficult to attain and sustain. Fifty, 100 and 200 NF per dwarf apple tree at every other tree and at every third tree were evaluated. The most practical release threshold, a compromise among the different parameters, was established at 100 NF per tree at 2.2 mites per leaf. At this threshold, biological control of phytophagous mites within the same season was highly probable, irrespective of the canopy of the trees touching or not touching one another.

Parallel with this study, another small-plot study showed that 140 g/ha of cyhexatin (Plictran® 50WP) (one fourth the Canadian label rate) could be used as a strategic treatment in biological control plots to tilt the prey-predator ratio in favour of the predator, whenever biological control of phytophagous mites by the predator seemed to be in difficulty. The cyhexatin treatment reduced the

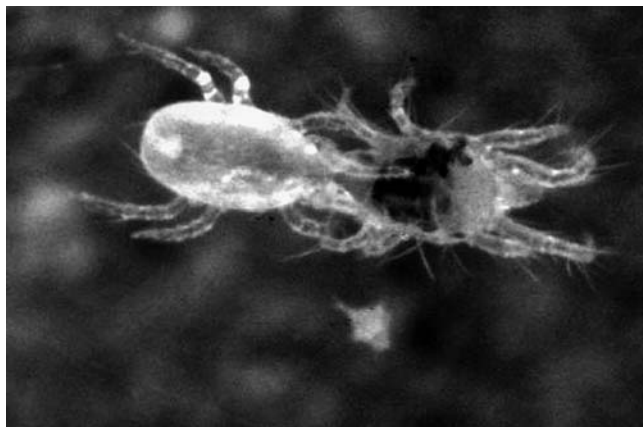


Fig. 41.1. An adult *N. fallacis* reared in a greenhouse feeding on an adult two-spotted spider mite (Photo by J. Lasnier).

prey population, but it did not eradicate it completely. Based on these results, the third study was initiated in an immaculately weed-free plot at the Agriculture and Agri-Food Canada research orchard at Frelighsburg, Quebec.

The third study

Prior to the commencement of this study, the block was sampled systematically throughout the season in 1978 to note if any indigenous phytoseiid predators were present. None were found, and in the spring of 1979, 10,000 NF were released in 100 dwarf apple trees. The predators were distributed on the scaffold limb section of the tree, on leaves with host mites present. The leaves were stuck to the scaffold limb with double-sided sticky carpet tape.

Phytophagous and predacious mites were sampled from early June to late September from ten leaves per sample tree per sample date. In 1979, biological control of phytophagous mites in the experimental orchard became a reality by late June. In subsequent years, the ratio of NF to prey was monitored and an index described by Croft and McGroarty (1977) was used to check whether biological control of phytophagous mites was taking place. Whenever required, cyhexatin was applied at a reduced rate and additional predators were released to tilt the predator-prey ratio in favour of the predator to re-establish biological control (Bostanian and Coulombe, 1986).

Other arthropod pests were managed by the application of azinphos-methyl, pirimicarb and phosmet. Apple scab, a scourge to apple growers in north-eastern North America, was managed by protective and 'after infection' curative treatments. The following fungicides were applied whenever required: captan, captafol, dodine and dichlone. At harvest, every year (1979, 1980, 1981 and 1983) 3000 apples were scored for injury caused by at least ten species of arthropods and scab. With the exception of 1981, the mean percentage of damaged fruit was less than 5%. A cost analysis showed that the programme was 34% less expensive than 'supervised chemical control', the most prevalent programme in Quebec at the time. In the supervised chemical control, pesticides were applied on regional monitoring information.

At this point, the study could have been considered complete. We had mastered the mass rearing of OP-resistant NF, eliminated acaricide treatments completely or had them applied at very low rates, estimated release numbers of predators per tree, reduced the rate of insecticide treatments to manage other arthropods, and decreased the cost of protection by 34%. The only step that remained was an extensive grower education in our procedures. However, we felt that before we embarked on such a grower programme, the entire procedure should be validated in a commercial context in blocks of at least 1 ha each.

The fourth study

Two blocks of 1 ha each were selected at 'Pommaraie de Dunham Inc.', Dunham, Quebec. A more modern rearing facility with automatic watering systems was set

up and predator production was doubled. At the appropriate action threshold, approximately 100,000 predators were released in each plot and predator–prey ratios were monitored carefully. After two seasons, the study was considered a partial success, terminated and shelved for ever. It was a very educational experience to the research team. For example, the action threshold (2.2 pest mites per leaf) to inoculate predators in an orchard was from a practical point of view difficult to respect. In 1988, just as the predators were to be released, adverse weather conditions forced the postponement of the release date by several days. Meanwhile, the trees had been treated with a ‘protectant’ fungicide against apple scab. As the fungicide was known to have deleterious effects on the predator, a delay of 3 weeks was respected before the release of the predators. In the meantime, the prey population increased and, even after several inoculations, biological control in the two blocks could not be achieved.

In 1989, the inoculations were done at the action threshold; a strategic treatment of 700 g/ha of propargite (Omite® 30WP) was also used to tilt the prey–predator ratio in favour of the predator. Biological control of phytophagous mites seemed to be working when NF migration towards the ground cover brought the study to a standstill. Field observations confirmed reports in the literature that the apple rust mite (ARM), *Aculus schechtendali* (Eriophyidae), was an alternate food for NF. However, we found NF to be a restless predator, and as the population of tetranychid mites decreased in the tree canopy, more and more NF searched for tetranychid mites, their preferred food on the ground cover. In the middle of summer, the trunks of trees resembled eight-lane super highways, with seven lanes of NF going towards the ground cover and one lane of NF going towards the tree canopy. This was because in commercial orchards, weed management was not as stringent as in small plots in Agriculture and Agri-Food Canada experimental orchards. Hence, if any weeds that harboured two-spotted spider mites (TSSM) *Tetranychus urticae* (Tetranychidae) were located by the restless predators, they would migrate and colonize the weeds. Meanwhile, a virtually non-existent tetranychid mite population in the tree canopy allowed residual ARM (the least preferred food of NF) to increase to very high densities (2000 ARM/leaf). Thus, while we had managed to control the tetranychids on the trees, ARM had now become a problem. The ARM could be managed with endosulfan (Thiodan® 50WP). However, this scenario, along with the complex logistics required to inoculate orchards with NF (100,000/ha), indicated the impracticality of the approach in a commercial context.

The Second Period

The 1990s to date: the era of biological control by conservation and augmentation

The emphasis of biological control for phytophagous mites from 1979 to 1989 had focused on inoculative releases. Such releases had been defined by DeBach (1964) as strategic releases of a biocontrol agent in large numbers where the released organism and its progeny would function as biocontrol agents.

Inspired by the interest shown by apple growers in biological control and some successes made by semi-organic apple growers in the late 1980s, we changed the research strategy from inoculation to conservation and augmentation. We had made a full circle and returned to Pickett's original approach of 1946. Conservation and augmentation meant the creation of an environment that would conserve biological control agents and allow them to increase in numbers. The extensive published and unpublished toxicological data (Bostanian *et al.*, 1984, 1998; Bostanian and Bélanger, 1985; Bostanian and Racette, 1997), along with the experience that we had acquired in recent years in biological control of mites, placed us in a very privileged position to create entire orchards that were friendly for the natural recolonization and propagation of predacious mites from the perimeters of commercial orchards. Another factor that helped us was the availability of sterol inhibitor and strobilin fungicides that were innocuous to predacious mites. This approach was initiated on less than 10 ha in 1991, and, by 2006, most apple orchards in Quebec had not been treated for mites for at least 5–10 years.

The predacious mites that control the pest species are *N. fallacis*, *T. caudiglans* (Phytoseiidae), *A. fleschneri* (Stigmaeidae), *Balaustium* sp. (Erythraeidae) and sometimes *A. baccarum* (Anystidae). *H. vitripennis* (Miridae) has also been noted in a number of orchards. We are currently trying to understand the coexistence of these species in commercial orchards. Orchards where biological control of mites was well established are now being used as natural nurseries. We have developed techniques to harvest the complex of predacious mites from these nurseries and introduce them into orchards where biological control of phytophagous mites by natural recolonization is being established. As the conservation and augmentation technique is based on a complex of predators, it is far more stable than the approach used in the early 1980s. Similar to the programme with 'inoculative releases', the growers have to follow a pesticide regime that is ecologically sound (Lasnier, 1993). In return, they save on acaricide costs and application time. To date grower interest has been keen because of the high cost of acaricides, resistance problems associated with mites and, last but not least, environmental concerns. A survey of pest control advisors working in pome fruit recently suggested that over 80% of the orchardists in Quebec have adopted our technique and were not treating for mites. The procedure to transfer mites, along with an exhaustive summary reporting the toxicity to predacious mites of most of the pesticides currently used in orchards, was brought to the attention of every grower across Quebec in 2002 (Lasnier *et al.*, 2002) and to interested growers across Canada in 2004 (Lasnier *et al.*, 2004).

Transfer of predacious mites from a donor to a recipient orchard

The recipient orchard block must be at least 0.2 ha in size and must provide sufficient pest mites for the beneficial mites to feed upon. If the pest mite population in the recipient orchard is high, chemical controls should be used to reduce the mite populations down to a manageable level prior to the introduction of predatory

mites. Failure to reduce the pest populations to a manageable level may result in unsatisfactory control, possibly causing leaf bronzing.

Wood from winter or summer pruning is used to transfer predatory mites from a donor orchard into a recipient orchard. Winter pruning should be carried out on 3-year-old or older wood, while summer pruning should be done on the annual growth and suckers. The amount of pruned wood collected and transferred to orchards depends upon the area of the recipient orchard, the population density of predacious mites on the pruned wood and the size of trees within that orchard.

Since predatory mites are sensitive to temperature extremes, desiccation and starvation, pruned wood should be handled with care. The pruned wood should be tied together in bundles of about 5 kg each and placed vertically and gently into a closed trailer or small truck to protect the predators from harmful winds during transport to the recipient orchard. In summer, this transfer should preferably be carried out on the same day as the collection. In winter, pruned wood should be transferred as soon as possible.

In winter, the bundles of winter-pruned wood should be placed vertically against the base of the tree trunks from bud break until petal fall to allow predatory mites to migrate into the orchard (Fig. 41.2). In summer, the pruned annual growth branches and suckers from the donor orchard are distributed in the recipient orchard. The branches are placed on the foliage of fruit-bearing branches.

After the release of beneficial mites in an orchard, growers must use pesticides that have minimal effects on predatory mites. Pesticides that are non-toxic to predators will allow beneficial mites to survive in orchards and provide natural control of pest mites. Predators that had survived pesticide treatments carried out in the donor orchard against insect pests and diseases are likely to be tolerant and resistant to these pesticides. Therefore, the owner of the recipient orchard should obtain the list of pesticides used in the donor orchard and use this information to plan his pest management strategies.

Finally, just as it is difficult to estimate the number of predators that may have been transferred from a donor to a recipient orchard, it is equally difficult to



Fig. 41.2. Research assistant placing a bundle of winter-pruned wood at the base of a recipient tree (Photo by J. Lasnier).

determine how long it would take to establish biological control in an orchard. It may require more than one season. Consequently, the population of pest mites may be high in the first season, but it eventually subsides to acceptable levels by the second or third season. Biological control is considered established when on average at least 10% of the leaves harbour a predator in early July.

Concluding Remarks

A highly robust, grower-friendly biological control programme for phytophagous mites in orchards is now in place across Quebec. It is based on the dominance of at least two predacious mite species per season and often another one to two predator species of minor importance. A key factor for this success is based on a regular comprehensive evaluation and understanding of the toxicology of pesticides that may be used in an orchard. Whenever possible, this information is generated in confidence with the cooperation of the agro-chemical industry. At the appropriate time, the information becomes public and it is relayed in a suitable format to growers, sometimes even before the scientific data is published in recognized journals. Armed with this information and the cost of treatments, growers, with the help of their pest control advisors, design their own management programmes and assume all risks.

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42

Management of Aphid Populations in Cotton through Conservation: Delaying Insecticide Spraying Has its Benefits

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Overview: Although natural biological control of pests occurs at almost all times and places, it frequently is insufficient to keep pests below the economic threshold. However, in some cases, naturally occurring biological control agents predictably hold pests in check, allowing the delay or avoidance of pesticide application. This is the story of one successful effort to utilize natural biological control of an insect pest, the cotton aphid, by predicting epizootics caused by the fungus, *Neozygites fresenii*.

The Cotton Aphid

The cotton aphid, *Aphis gossypii* (Homoptera: Aphididae), has been a perennial secondary pest of cotton worldwide. Insecticides applied to cotton for pests such as bollworms, plant bugs, stink bugs, the boll weevil and other pests kill predators and parasitoids that help keep aphid populations in balance, thereby flaring aphid populations. This was noted especially during the 1940s when calcium arsenate was used for boll weevils, and again during the 1980s and 1990s when organophosphates and pyrethroids flared aphids. High aphid populations (Fig. 42.1) lower cotton yields by stunting plants, adversely affecting fruit development, by lowering boll weight and reducing square and boll retention, and by causing 'sticky cotton'.

Each year in the USA about 35,000 farms are planted to 13 million acres of cotton, producing about 17 million bales (each bale weighs about 500 pounds) with a total value of \$40 billion to the industry and a retail value of \$120 billion. Consequently there is great interest and economic gain in finding cheaper solutions for cotton aphid management.



Fig. 42.1. Cotton aphid populations on cotton can become extremely high.

How It Started: the Importance of Field Observations

In October 1989, I began work as an insect pathologist at the University of Arkansas. As soon as I arrived, our cotton entomologist, Dr Phil Tugwell, brought me cotton leaves frozen in plastic bags that he had collected from aphid-infested cotton fields during the past summer. He told me, 'Don, something is killing cotton aphids in cotton fields. Maybe you can determine what it is.' His field observations are an example of the value of the 'well-calibrated eyeball'. I examined the leaves and found, unfortunately, that they were overgrown with saprophytic fungi and no pathogen could be identified (Fig. 42.2). During the next cotton season, in 1990, I went with Dr Tugwell to cotton fields during aphid population declines, collected fresh material and discovered that the aphid populations were being decimated by *N. fresenii* (Zygomycotina: Entomophthorales: Neozygiteaceae), an obligate fungal pathogen of aphids (Steinkraus *et al.*, 1991). The keen observations of Dr Tugwell combined with my expertise in diagnosis of insect pathogens are examples of Louis Pasteur's famous quote, 'Chance favours the prepared mind.'

Founded on Basic Research

The observation that the fungus *N. fresenii* was decimating cotton aphid populations led to years of basic research on the epizootiology of this pathogen. Billions of aphids across vast cotton fields, counties and states were being killed by this pathogen. Often within 1 week, plants went from being covered with live aphids (Fig. 42.1) to almost 100% killed by *N. fresenii* and overgrown with saprophytic fungi (Fig. 42.2). We were interested in how widespread the epizootics were and therefore surveyed cotton fields intensively in Arkansas and nearby states. We developed techniques to efficiently diagnose large numbers of aphids to accurately determine prevalence of the fungus in aphid populations (Steinkraus *et al.*, 1995). After several years of research, we realized that the epizootics were very widespread in the mid-southern states of Arkansas, Louisiana, Mississippi and Tennessee, and the south-eastern states of Alabama, Florida, Georgia, North Carolina and South Carolina. We found that the epizootics occurred regularly each year

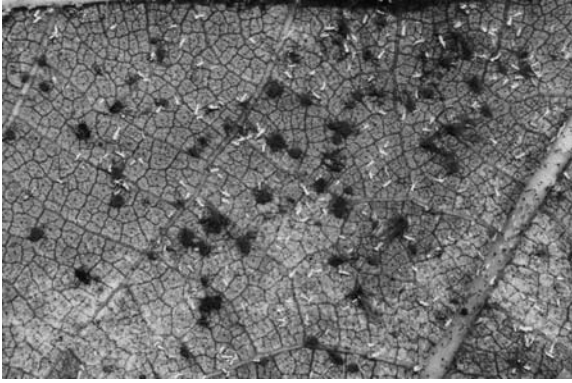


Fig. 42.2. *N. fresenii* epizootics frequently kill nearly all cotton aphids in a cotton field within a few days. All that remains are dead aphids covered with saprophytic fungi.



Fig. 42.3. Aphid-infested cotton leaves are collected from cotton plants and placed into vials containing 70% ethanol.

within a 5-week period during June and July, and that once epizootics began, aphid populations declined to economically unimportant levels within about 7 days (Steinkraus and Hollingsworth, 1994; Hollingsworth *et al.*, 1995).

The Importance of Grower/Industry Interest

Of key importance was the intense interest of the cotton-growing community in reducing input costs for pest control. My initial research describing *N. fresenii* as the causal agent of the epizootics was presented at the annual Beltwide Cotton Conference, which brings together several thousand members of the entire cotton industry, from growers and extension agents to scientists, engineers and textile industry people. After my talk, I was approached by Dr Bob Nichols, a Senior Director of agronomy research at Cotton Incorporated (CI), a cotton-industry-funded institution that increases the demand for and profitability of cotton through research and promotion. He told me that CI was interested in my research and wished to fund it.

From the beginning we publicized our research on cotton aphids in farm journals, such as *Cotton Grower* and *Delta Farm Press*, and extension publications,

and spoke at grower meetings. The cotton-growing community is made up of alert, intelligent people, and within a couple years almost all growers were familiar with *N. fresenii*, the aphid fungus, and they eagerly awaited its arrival each season.

Taking Advantage of Natural Control of Cotton Aphids: How the Cotton Aphid Fungus Sampling Service was Born

After a few years of research, we realized that we could benefit cotton growers by informing them of the fungus prevalence levels in individual fields. Therefore, in 1993 we began offering the cotton community a free service. We diagnosed aphid samples from their fields, providing them with the fungus prevalence level and suggesting that if the fungus level in a field was 15% or higher, they could postpone insecticide applications for aphids and let the fungus reduce the aphid population. This has directly reduced input costs, a major goal of current cotton research. At first the service was offered only to Arkansans, but due to demand from other states we gradually added all the cotton-growing states in the mid-south and south-east: Alabama, Florida, Georgia, Louisiana, Mississippi, North Carolina and South Carolina.

Each year during April and May, we contact cotton extension agents, consultants, cotton growers and researchers in each state by e-mail, fax or telephone and ask them if they would like to participate in the service. New participants can also sign up on our website (<http://www.uark.edu/misc/aphid/>). We try to make the entire process as simple and easy for participants as possible. If they agree, they are sent a sampling kit. Kits consist of vials filled with 70% ethanol, wrapped in paper towels, and placed in cardboard mailing tubes. Data sheets are also included, along with pre-addressed return FedEx envelopes. The participants are requested to sample aphids in their cotton fields by placing infested leaves (Figs 42.1 and 42.3) into the vials. Participants fill out data sheets indicating the field location and size, aphid infestation level, and other aspects of the sample field, and whether they are considering spraying for aphids soon and therefore need the results rapidly. Aphid samples usually begin arriving during mid-June and continue until epizootics have eliminated most aphid populations, usually during mid-July.

From each field, we diagnose a randomly chosen subsample of 50 aphids. Aphids are diagnosed by trained hourly personnel hired for the season. They squash the aphids carefully on microscope slides (Figs 42.4 and 42.5) and diagnose each individual aphid, recording the stage of infection. The prevalence of the fungus in the field is then determined and faxed or e-mailed back to the participant. We attempt to provide this information to the grower within several days of receiving the sample. All the data is also posted daily on our website, which contains archived data for all states back to 1999 and information for potential participants on how the service operates and how to interpret prevalence levels.



Fig. 42.4. Random subsamples of cotton aphids from the field are squashed in lactophenol for diagnosis.



Fig. 42.5. We diagnose 50 aphids from each sample, five aphids per coverslip, with a phase microscope at 200x. Cotton aphids are small, soft, easily mounted and squashed on slides, making precise diagnosis possible for each aphid.

Value of the Cotton Aphid Fungus Sampling Service

The service utilizes a fundamental principle of integrated pest management (IPM). In the original definition of IPM by Stern *et al.* (1959) they stated, 'Chemical control should be used only when the economic threshold is reached and when the natural mortality factors in the environment are not capable of preventing the pest population from reaching the economic injury level.' This is still good advice and is the theoretical basis of the Cotton Aphid Fungus Sampling Service.

It is difficult to place a precise value on this service. We directly help many growers and researchers who send in samples. From their comments, it is clear that in many instances, the service saves individual growers many thousands of dollars. This comment from a consultant makes this clear, 'We had five growers prepared to spray ca. 2500 acres until the service revealed that the fungus was present. At \$7.50 per acre, this was a significant saving' (\$18,750). When this is multiplied by the number of fields each year in which the service detected epizootics, the direct value has been in the hundreds of thousands of dollars. However, the indirect value of the service is much greater. Because of our successful efforts at publicizing the biological control provided by *N. fresenii*, almost all consultants, growers and extension agents are aware of when the fungus is found in their counties and areas. Data from every sample is placed on our internet site each day. Extension agents and others check our website, and when we detect fungus,

they publicize it in their e-mail newsletters and visits with growers. By this means, thousands of cotton growers take advantage of the service. Therefore, in many years the fungus has saved growers in the mid-south and south-east millions of dollars in input costs.

Special Aspects of the *N. fresenii*/Cotton Aphid System

There are a number of reasons why this project is successful. Cotton aphids are very small and soft bodied, which permits large numbers to be easily collected and preserved in 70% ethanol. Consequently, it is easy to ship reasonable sample sizes to our diagnostic laboratory. Aphids are easily squashed on microscope slides, liberating fungal protoplasts, hyphal bodies and resting spores from the aphid's body, making diagnosis accurate (Figs 42.6 to 42.8). It would be very difficult to do a similar diagnosis with large numbers of larger insects because whole insects could not be squashed on a slide and all their organs and contents examined. Another special feature of this system is the fact that nearly all aphids found reproducing on cotton in the USA are *A. gossypii*. Other aphids are relatively rare, so our technicians do not have to constantly identify aphids to species. In addition, the most prevalent pathogen found infecting *A. gossypii* in cotton is *N. fresenii*. We occasionally find *Pandora neoaphidis* infecting aphids, but it is readily distinguished from *N. fresenii*. The diagnosis process is simplified because it is a one host/one pathogen system. It is also important that *N. fresenii* fungal structures are highly distinctive and persistent. For instance, the relatively large capilliconidia (infective secondary spores) are glued on to the host legs, antennae and other integumental areas with a strong fungal 'glue', which causes the spore to adhere at right angles to the host and remain attached to the host even after years in ethanol. Therefore, when the distinctive 'football'-shaped capilliconidia are seen attached to the leg or antenna of an aphid, it is easily diagnosed (Fig. 42.6).

There are biological reasons why this service has succeeded. Cotton aphids are parthenogenetic and reproduce very rapidly. Between 1989 and 2006 many cotton fields were infested with large numbers of aphids, resulting in regular interest in the service. Second, *N. fresenii* is an amazingly efficient natural pathogen with a very rapid life cycle. From the time an aphid contacts an infective spore



Fig. 42.6. Leg of a cotton aphid, showing the distinctive and firmly attached capilliconidia of *N. fresenii*. Capilliconidia are the infective stage of *N. fresenii*.

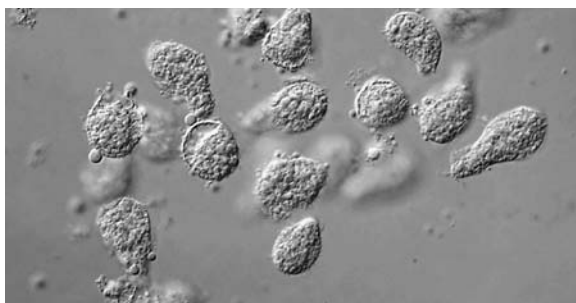


Fig. 42.7. When infected aphids are squashed, protoplasts or hyphal bodies of *N. fresenii* are released from the haemocoel. These can be differentiated from host cells.

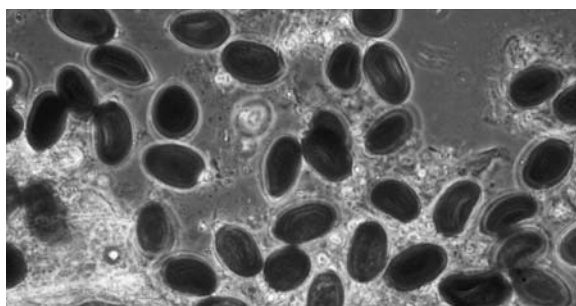


Fig. 42.8. Resting spores of *N. fresenii* liberated from the haemocoel of an infected cotton aphid. This project has been greatly simplified by the distinctive structures of *N. fresenii*.

until the aphid's death is usually 3–5 days. There is also no dose response: one infective capilliconidium is sufficient to infect an aphid. Each infected aphid produces about 3000 primary conidia, which are explosively discharged on to a leaf or into the air. This results in large numbers of primary conidia directly contacting adjacent aphids on a leaf for efficient short-range transmission. During an epizootic it also results in enormous numbers of primary conidia floating through the air within and between cotton fields. Sampling air over cotton fields during epizootics showed that the numbers of primary conidia per cubic metre of air could reach more than 90,000, and about 50% of healthy sentinel aphids placed within cotton fields during epizootics became infected overnight (Steinkraus *et al.*, 1999). The fungus (by an unknown mechanism) kills the host at about 22:00 h and discharges its spores between about 24:00 h and 06:00 h, when relative humidity is high and the conidia survive well. These biological aspects – rapid life cycle, one spore sufficient to infect a host, large numbers of spores produced per host, spores discharged from the host on to other hosts and into air – combine to result in epizootics, which frequently reduce aphid populations to negligible levels within a week of epizootic onset. A sampling service like ours would be more difficult or impossible with natural enemies with long life cycles and large hard-bodied hosts.

Difficulties and Problems with the Service

There have been several difficulties associated with this service. First, each year we need to hire and train 4–5 skilled hourly workers. While we have usually been able to find excellent workers, the work flow for the service is somewhat difficult

to manage. This is because samples are not shipped to us at constant intervals. Growers only collect aphids when fields become infested to the point that they are concerned. This generally happens with some synchrony at similar latitudes. Therefore, during some weeks we receive many more samples than we can easily handle and in other weeks fewer. Very few samples come in before mid-June or after late July, therefore other tasks must be found to keep employees busy while waiting for samples.

Second, it takes 1 h to process one aphid sample. We take a random subsample of 50 aphids, squash them on slides, diagnose each aphid at 200x with a phase contrast microscope, and then send the results to the participant. In most years, we receive samples from about 300 fields and this is about as many as we can handle. In order to process more samples, we would need to hire additional people and buy more microscopes, and we have neither the funds nor the space to do so.

A third problem is funding. Cotton Incorporated has been very supportive of this programme, providing \$26,000 per year. This support is crucial. It pays for the 4–5 hourly workers, slides, coverslips, nail polish, FedEx charges and other associated expenses. Additional funding for the basic research came from USDA competitive grants and the Arkansas Experiment Station. However, funding has been a yearly source of concern.

The Cotton Aphid Fungus Sampling Service has now operated for 14 continuous years. In 2004 and 2005 cotton aphid numbers were relatively low across the cotton belt. Changes in cotton production appeared to have reduced aphid problems. Cotton aphids are often induced secondary pests. The implementation of boll weevil eradication and Bt cotton result in fewer insecticide sprays, hence less flaring of cotton aphids. New inexpensive and highly effective insecticides, such as the neonicotinoids, also greatly reduce aphid infestations. Concurrent with these agronomic changes, increased numbers of beneficial predators and parasitoids have been surviving in cotton fields, helping to hold aphid numbers down. As a result of all these factors, in 2004 and 2005 fewer cotton fields were heavily infested with aphids. When this occurs, the production of migratory alate aphids also declined. With fewer alate aphids fewer new fields are colonized. As a result we began to think that the need for the sampling service had ended. But in 2006, cotton aphids were again a major problem for cotton growers. Extension agents think that the cotton aphid has begun to develop resistance to the neonicotinoids. Many samples were received by the service, and growers, extension agents and consultants were interested in when the epizootics would provide some help controlling cotton aphids.

Rewards of the Service

This project has been rewarding on many levels. First, there have been purely scientific rewards. We were able to conduct in-depth, long-term biological and epizootiological studies in the laboratory and field on *N. fresenii*. Our basic research provided the foundation for our applied research and has improved our understanding of how and why *N. fresenii* epizootics are so widespread and rapid acting. A second reward has been the satisfaction of providing practical help to

cotton growers. In most cases, insect pathogens have been difficult to utilize in large-acreage/heavily managed crops such as cotton. Therefore, it has been exciting that we have been able to educate cotton growers about a fungal pathogen and they have been able to save input costs by paying attention to an entomopathogen. A third reward has been the opportunity to work with many first-class agricultural scientists, farmers, extension agents, consultants and research assistants on this project.

In summary, this has been a fascinating project. It has been rewarding in terms of biological research on the epizootiology of an insect pathogen as well as rewarding in practical help for cotton growers to reduce their input costs.

Acknowledgements

This project has benefited greatly from the input of many individuals, too numerous to name individually. Dr Patricia O'Leary of Cotton Incorporated has been a major supporter of this service. My research assistant, Gabriele Boys, (now retired) patiently diagnosed many thousands of aphids and taught others how to do so. Jon Zawislak designed and maintains our website. Many thanks to the many hourly workers who have squashed aphids on slides for the past 14 years. Special thanks to Dr Gus Lorenz and his extension colleagues in all the states for their invaluable support. This project has been funded by Cotton Incorporated and the Arkansas Experiment Station.

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43

Management of Pests and Diseases in New Zealand and Australian Vineyards

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Overview: ‘What good are all those species that man cannot eat or sell?’ (Odum, 1971). This chapter considers this question in the context of pest and disease control in Australasian vineyards. We show that species of insects and microbes that cannot be eaten or sold can have value to vineyard managers by providing tangible ‘ecosystem services’. Predatory and parasitic arthropods can help suppress insect pests whilst microbes, especially fungi, which are encouraged by mulching, can help reduce the severity of vine diseases.

Introduction

The opening question sums up the typical view of humans regarding the species that share planet Earth. It was posed by the ecologist Odum (1971) in his book *Fundamental of Ecology*, and since this time researchers around the world have been investigating whether such species have real value. Such work led to the publication of a detailed analysis of the ‘ecosystem services’ provided by such species, in which it was estimated to be worth \$33 trillion per year (Costanza *et al.*, 1997). These services include insects that pollinate crops or kill pests, vegetation that helps purify water and forests that take carbon dioxide from the air. For insects, the economic value of wild species (i.e. excluding cultured species such as silkworms) has recently been estimated to be at least US\$57 billion per year in the USA alone (Losey and Vaughan, 2006).

This case study is about one aspect of such ecosystem services, the control of pests and diseases using naturally occurring beneficial species. When the term ‘biological control’ is used, people tend to think of cases where a species is released into a new continent, where it spreads out and helps suppress the target weed or plant-feeding insect. This ‘classical’ approach to biological control comes with risks, however. Most people have heard of cane toads, which were released

in Australia in the mid-1930s to control cane beetles. Not only did the toads fail to do this, to the present day they are dispersing across tropical Australia, feeding on native species, out-competing native amphibians and – because they produce toxins that Australian wildlife is not familiar with or adapted to – are poisoning predators as diverse as fish and birds. There are other cases of such ill effects resulting from exotic biological control agents in Hawaii and mainland USA. Though it will be clear from other case studies in this book that much more care is taken in contemporary attempts at classical biological control, there is an alternative that avoids the risk of such off-target impacts altogether.

Conservation biological control (CBC) seeks to make better use of existing beneficial insects rather than release exotic species (Barbosa, 1998). The aim of this chapter is to review CBC work in one particular agricultural system – vineyards – to illustrate the types of factors that influence levels of success and the practical barriers that may slow adoption.

Conservation Biological Control in Vineyards to Enhance Natural Enemies of Insect Pests

Biological control of insect pests in New Zealand and Australian vineyards is focused largely on the management of leafrollers, especially the light brown apple moth (LBAM), *Epiphyas postvittana* (Lepidoptera: Tortricidae), which is widely considered to be the most serious pest of grapevines. The larvae of *E. postvittana* damage grapevines by feeding on new shoots, flowers, berries, stalks and leaves. Crop loss is also caused by the larvae exacerbating infection by the fungus *Botrytis cinerea*, leading to bunch rot disease in damaged bunches. Such damage may be significant. In New Zealand, for example, mid-season losses as a result of bunch rot may exceed 20%, and complete loss of crops can occur before harvest in very wet seasons.

Currently, the most common method of control of insect pests in vineyards is use of insecticides. However, as New Zealand wines are being marketed as ‘the riches of a clean, green land’ and the negative effects of pesticides are becoming more widely known, alternative methods of managing insect pests are being studied. Similar market pressures apply in Australia, with, for example, major wine companies requiring growers to keep ‘spray diaries’ and adhere rigidly to recommended pesticidal products and spray schedules. Alternative methods for controlling insect pests include the use of pheromone disruption and *Bacillus thuringiensis* (Bt) sprays, as well as growing levels of interest in arthropod biological control agents.

Conservation biological control (CBC) involves manipulation of the environment to enhance natural enemy populations and to increase their effectiveness at controlling the target/pest organism (Gurr *et al.*, 2004). The practice of CBC frequently incorporates flowering plants into the agro-ecosystem to enhance the populations and fitness of natural enemies by providing them with resources which may have been previously absent or scarce. This paucity of non-crop vegetation is evident in the description of one part of New Zealand – the Canterbury

Plains – as ‘*Lolium* and *Trifolium* laid out like green linoleum’. Flowering plants are important for the activity of natural enemies in agricultural systems because they provide resources such as food (in the form of pollen and/or nectar), shelter and/or alternative prey/hosts (Landis *et al.*, 2000). Many studies have demonstrated that providing adult parasitoids with a sugar source increases their lifespan and egg-laying capacity. Clearly, this offers scope to boost their impact on pest populations.

Conservation biological control is a relatively new branch of pest management, so not all research programmes meet with complete success. How then, can CBC success be measured? Gurr *et al.* (2003) propose the following hierarchical system.

1. Aggregation of agents at or near the flowers.
2. An enhancement of the agent’s ‘fitness’ (longevity, fecundity and searching efficiency).
3. An increase in parasitism or predation rate in the pest population.
4. A decrease in pest population density.
5. The pest populations are brought below the relevant economic threshold (so avoiding the need to apply curative insecticides).

In this case study we use the vineyard system to discuss the levels of success that have been achieved in understorey management in vineyards in relation to managing populations of leafrollers such as *E. postvittana* in New Zealand and Australia.

Levels of Success Reached by Planting Flowering Plants in Vineyards

The key pest of vineyards, *E. postvittana*, is attacked by a wide range of parasitoids and predators in Australasia during most of its developmental stages. However, it is the braconid wasp *Dolichogenidea tasmanica* which is the most common parasitoid attacking leafroller larvae in New Zealand. This parasitoid is also important in Australia, along with the tachinid fly *Voriella uniseta* and a range of predators such as the pentatomid bug *Oechalia schellenbergii* and the larvae of lacewings such as *Micromus tasmaniae*. Egg parasitoids, especially the Australian endemic wasp *Trichogramma carverae*, are significant, and *T. carverae* has been commercialized as an agent available for mass release for *E. postvittana* control.

New Zealand studies by Lisa Berndt and Nic Irvin (Irvin *et al.*, 2000) have highlighted the scope for conservation biological control techniques to enhance natural enemy populations of *E. postvittana* in vineyards. The first level of the hierarchy of research outcomes (see above) was met when significantly more male *D. tasmanica* were collected on yellow sticky traps in buckwheat plots compared with control plots where no flowering plants were present. Buckwheat did not appear to increase local adult parasitoid populations, and the low numbers of parasitoids captured overall may explain this.

In a laboratory study, the effects of buckwheat plants on *D. tasmanica* fitness were investigated. Results showed that longevity of female *D. tasmanica* was increased from 12 days (water only) to 35 days when they were exposed to buckwheat, and that buckwheat enhanced potential fecundity by 62%; thus the second level of the hierarchy was reached. The third level was achieved when parasitism rates of leafroller larvae were increased by more than 50% in one vineyard out of three when buckwheat flowers were present. At the other two vineyards, buckwheat had no effect on parasitism rates, but at these locations, leafroller populations were low, because insecticides had been used in that growing season. This illustrates one of the difficulties inherent in field testing CBC methods within production systems where pesticides are standard practice.

Conservation biological control is aimed at assisting natural enemies, but what about the possibility that pests may also derive benefit from flowers? An example of this danger was provided in Australian studies where flowers such as buckwheat were sown in the margins of potato fields and pest moths flourished (Baggen and Gurr, 1998). To avoid this problem, recent work in Australia by Mahmuda Begum not only investigated which food plants enhanced *T. carverae* but which were not fed upon by *E. postvittana* larvae and did not provide nectar to adult moths.

Although the research described above has shown that the first three levels in the hierarchy of research outcomes can be achieved in the vineyard system, it is only in very recent field studies by Sam Scarratt and colleagues (Scarratt *et al.*, 2004) that the fourth level has been demonstrated. Current research is addressing whether the reductions in pest density achievable by this approach will adequately suppress pests: the fifth and final level in the hierarchy.

Industry Shows Interest

Despite the fact that CBC in vineyards is far from being a well-honed science, already industry members have shown a significant level of interest, especially in New Zealand. There, government funding (Foundation for Research Science and Technology) is contributing to a 'greening of the Waipara' initiative, in which vineyards are hosting large-scale experimental evaluations of vineyard ground covers such as buckwheat and phacelia.

In Australia, industry consultation has indicated that ground-cover plants may be acceptable, provided that they did not interfere with air flow, especially beneath vines, which is important for reducing damage from fungal diseases (see below) and frost. The requirement for prostrate plants favours the use of alyssum, which – fortuitously – has been shown by experimental studies to enhance the longevity and fecundity of the key natural enemy, *T. carverae*, without benefitting adult or larval *E. postvittana* (Begum *et al.*, 2006). Industry members in Australia also favour positioning such plants immediately beneath vines (rather than in the strip between vines), where they offer scope to suppress weeds that otherwise require labour-intensive mowing or carefully managed herbicide applications. Even the more direct competition between vines and ground covers that this arrangement leads to is viewed positively as a means of 'de-vigouring' the vine, thus

improving the quality of the resulting wine. These broader, non-insect, pest-related factors may prove to be important in favouring the uptake of this technology by vineyard managers, but there is also another potentially significant advantage of manipulating the floor of vineyards: plant diseases.

Plant Disease Management

Conservation biological control strategies set in place for the control of arthropods such as *E. postvittana* may also have important effects on plant disease. Botrytis bunch rot disease causes off-flavours, increased sensitivity to oxidation and other biochemical changes to the wine, and in temperate climates can cause total crop loss. In grapevines, *B. cinerea* typically overwinters on vine debris, and the resulting inoculum can contribute up to 70% of disease levels on the vine at flowering and 28% at harvest.

B. cinerea has traditionally been managed by improving canopy aeration via rootstock and trellis selection, pruning and leaf plucking; sanitation practices to remove *B. cinerea* inoculum; and chemical control. Increasingly, grape growers are under pressure to practise more sustainable, environmentally aware disease control owing to the increasing resistance to fungicides and a more discriminating global market place. Disease management through CBC is a new area of research in horticultural crops so literature on the area is limited. It is clear, however, that the encouragement of microbial communities that break down the inoculum of economically important diseases is of central importance. It is, therefore, analogous to the encouragement of beneficial arthropods to keep insect pest populations in check.

Early attempts to use mulches comprising vine prunings, grape marc (the residue left after pressing), green waste, pine bark, animal manure and mussel shells had an inconsistent effect on the severity of *B. cinerea*, though they tended to elevate numbers of soil fungi under the mulch and increase soil organic matter and potassium levels. Similarly inconsistent results were found in *B. cinerea* levels in French vineyard studies using mulches comprising household waste, straw, fresh bark and bark compost. A bark and sewerage-sludge compost increased degradation of boysenberry debris and reduced *B. cinerea* sporulation in laboratory conditions in New Zealand.

More recent work in New Zealand has used mulches to enhance soil microbial activity and degradation of vine debris to reduce *B. cinerea* inoculum on the debris itself (Jacometti *et al.*, 2007). At 50% flowering and leaf plucking, all mulch treatments significantly reduced *B. cinerea* primary inoculum on vine debris compared with the control (Fig. 43.1). The largest reductions were under anaerobic and aerobic grape marc and shredded paper, resulting in a three- to 14-fold reduction in conidiophore coverage, whereas grass clippings reduced conidiophore coverage two- to threefold, compared with the control. Degradation of vine debris was significantly increased by mulch treatments compared with the control. The two grape marc and paper treatments resulted in the greatest weight loss, which was a reduction in debris weight by up to three times compared with the control. Grass clippings were less effective, significantly reducing weight of the debris at leaf plucking by 1.5 times but not at 50% flowering. Levels of *B. cinerea* on vine

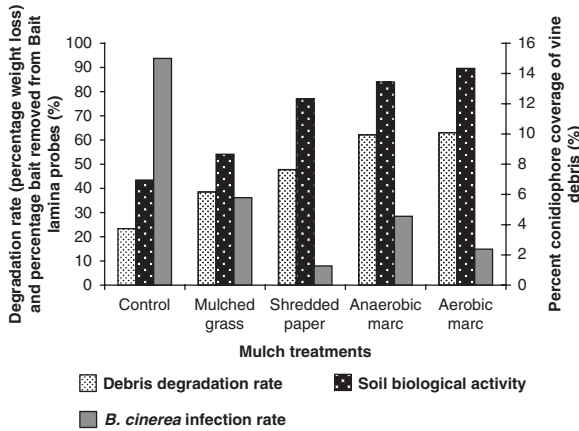


Fig. 43.1. Degradation rate, soil biological activity and *B. cinerea* infection of vine debris buried under organic mulches in an organic vineyard in Blenheim, New Zealand.

debris, degradation rate and soil biological activity under the mulch treatments were all significantly correlated at both assessment times. Again, the two grape marc and the paper treatments had the highest effect, with rates of biological activity under these treatments 2.5 times higher than those in the control. Under grass clippings, the soil biological activity was significantly elevated by a factor of 1.3 at leaf plucking.

Conclusion

Biological control of fungal inoculum via the enhancement of soil biological activity and vine debris degradation had not previously been demonstrated under mulches in a vineyard environment and highlights the potential for conservation biological control of plant diseases in horticulture. This illustrates the power of simple cultural practices to contribute towards plant disease problems. The biological mechanisms that operate in the vineyard system are potentially transferable to other horticultural systems and to other important diseases such as powdery mildew (*Uncinula necator*) and downy mildew (*Plasmopara viticola*). As this system seems to be driven by soil moisture and nutrient content, it could easily be combined with the use of flowering plants that help boost the activity of natural enemies of insect pests. For example, mulching these plants into the below-vine area after pruning would convert the plants that had supported natural enemies earlier in the season into a mulch that speeds breakdown of fungal inoculum on prunings over the winter.

As always, one of the measurements of success of conservation biological control is the uptake of the technology by industry. The research-based application of mulch or cover crops is a technology that could be easily integrated into current horticultural systems as it is inexpensive and of low risk. The use of some fungicides can be integrated into such a system as the fungicide is unlikely to penetrate the mulch and so the system will continue to operate. This technology has the potential to enhance ecosystem services, wine marketing and sustainability in vineyards by using organic waste to help disrupt the life cycle of *B. cinerea* and

potentially of other vine pathogens, an increasingly relevant technique in the light of consumer requirements for a 'cleaner and greener' product.

Overall, these cases of managing vineyard plant protection problems with ecologically based methods highlight that the importation of exotic biological control agents and the use of synthetic pesticides – each of which has potential hazards – may not always be necessary. Maybe a lot of 'those species that man cannot eat or sell' are more useful than has been realized. The challenge before us is to learn how to harness them to maximize the ecosystem services, such as pest and disease control, that they can provide.

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44 Take-all Decline: Model System in the Science of Biological Control and Clue to the Success of Intensive Cropping

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Overview: Take-all is a root disease of wheat and barley caused by the soil-borne ascomycete, *Gaeumannomyces graminis* var. *tritici*. The fungus causes serious limitations to grain yield when wheat is grown for 2 consecutive years in the same field. Crop rotation, preferably breaks of 2 or more years away from wheat or barley, was the only method recommended for reliable control. The discovery that this disease declines in severity and can all but disappear where wheat is grown in the same field over many years provided an exemplary model system for studying soil ecosystems that become 'disease suppressive'. This chapter summarizes the results of 40 years of research that led to the understanding of the key biological and biochemical components responsible for creating take-all suppressive soils and the resultant take-all decline. The chapter provides approaches to unravelling the complex microbial ecosystems in the rhizosphere and gives directions for management of root diseases in intensive cropping systems heretofore considered to be only controllable by broad-spectrum biocides.

Introduction

It was with both a sense of amazement and a tinge of pride that I looked over the audience of some 200 in attendance at the symposium 'The Nature and Application of Biocontrol Microbes III. *Pseudomonas* spp.' at the 2005 annual meeting of the American Phytopathological Society in Austin, Texas; amazed that so many plant pathologists were still interested in this group of biocontrol microorganisms and proud that four of the eight invited speakers were alumni or current leaders of the Pullman team working to understand and exploit take-all decline. Take-all is a disease caused by a soil-borne ascomycete, *Gaeumannomyces graminis* var. *tritici*, that develops on the roots and stem bases of wheat and on the roots but rarely stem bases of barley. It stunts early plant development, accelerates plant maturity later in the season and can seriously limit yields, sometimes to the extent that it 'takes all' the crop. 'Take-all decline' is the spontaneous remission of disease following continuous monoculture of wheat and barley. This phenomenon

has become a model system in the science of biological control of soil-borne plant pathogens. In fact, I will suggest that as a scientific milepost in rhizosphere microbiology the story of take-all decline today is approaching the sophistication and scientific interest of another beneficial plant-microbe interaction, namely that of *Rhizobium* biology and associated nitrogen fixation.

One year earlier, I was an invited speaker at the 4th International Crop Science Congress in Brisbane, Australia, with the assigned topic: 'In Defence of Continuous Crop Monoculture'. There is almost no end to the list of successful crop monocultures globally, including the decades-long establishment of managed turf and other perennials. However, very little has been done to reveal why they are successful production systems. Arguably, research into take-all decline is opening an entirely new way of understanding the success of, and opportunities for, continuous crop monocultures specifically and intensive cropping more generally.

One could say that my interest in biological control was by default, that I had no other options to control root diseases of wheat, but this would not be correct. It is true that options for root disease control in modern wheat-based cropping systems were and remain very limited. The traditional 3-year (and longer) crop rotations promoted in cereal-producing areas during the 20th century as the best means to control root diseases (Cook and Veseth, 1991) are now being replaced increasingly with intensive cereals, typically wheat 2 years in 3 or every year. The amount of land under clean tillage continues to decline and 'no-till' (direct-seed) systems, where the crop residue is left on the soil surface as a trashy seedbed, continue to increase. Some growers in the Inland Pacific Northwest made their transition to direct seeding less risky by burning the wheat straw prior to seeding wheat, but public pressure has greatly limited the use of this practice. Soil fumigation provides the most spectacular increases in crop growth response for wheat and barley, especially for fields most frequently cropped to cereals (Cook and Veseth, 1991), but is not affordable for wheat. The most ideal control would be to have cultivars with resistance to take-all, but genes for resistance to root diseases remain essentially unknown in the pool of germplasm available to wheat breeders.

My job, starting in the mid-1960s, was to provide the science and technology needed to limit or eliminate the yield-depressing effects of root diseases on wheat (and later barley) while depending little or not at all on traditional crop rotations, tillage, stubble burning, chemical pesticides or host-plant resistance. Indeed, disease suppression by soil microorganisms antagonistic to the pathogens was about the only option left.

My days as a graduate student at the University of California, Berkeley, from 1961–1964 with W.C. Snyder, K.F. Baker, S. Wilhelm, M.N. Schroth, T.A. Toussoun and A.R. Weinhold, all world leaders in research on ecology and control of soil-borne pathogens, prepared me to consider biological control by disease-suppressing soil microorganisms as the first line of defence against soil-borne plant pathogens. This philosophy was the foundation for the *International Symposium on Factors Determining the Behavior of Plant Pathogens in Soil*, held on the Berkeley campus in April 1963, and published in 1965 as *Ecology of Soil-borne Plant Pathogens: Prelude to Biological Control* (Baker and Snyder, 1965). I attended that symposium as a graduate student, and the proceedings, published the year I was hired by the USDA, Agricultural Research Service (ARS) and stationed at

Washington State University, Pullman, became the foundation for my work on ecology and biological control of soil-borne plant pathogens of cereals in the US Pacific Northwest.

My decision to focus on biological control in soil and the wheat rhizosphere was sealed when, in 1969, the late Kenneth F. Baker invited me to join him as co-author of the book, *Biological Control of Plant Pathogens* (Baker and Cook, 1974). It took 5 years to write that first book, which also meant 5 years of thinking deeply about biological control of plant pathogens with Ken Baker, an awesome opportunity for someone in their early thirties. I also made a conscious decision during that period of my career that I was not going to only write about biological control of plant pathogens, I was going to do biological control – of wheat and barley root pathogens.

Initiation of Take-all Decline with ‘Starter’ Soil

By the time I began my work in 1965, take-all was already well known for its severity on ‘back-to-back’ wheat in the US Pacific Northwest. Crop rotation, preferably breaks of 2 or more years, was the only method recommended for its control, and incidentally, is still the best method of control (Cook and Veseth, 1991). Indeed, conventional wisdom among plant pathologists in the region at that time was that, because crop rotation is so effective, no further work was needed on take-all. My question was: if crop rotation is so effective, why is there so much take-all? There were, and still are, many economic, environmental and ecological reasons why farmers around the world not only risk second and third consecutive wheat crops and even continuous cropping with wheat or wheat/barley sequences but now specialize in intensive wheat-based cropping systems if not continuous wheat monoculture. For me, there was the personal challenge: we know the effectiveness of rotations in managing soil-borne plant pathogens, but managing them in crop monoculture, while taboo to those that espouse a certain philosophy of ‘sustainable agriculture’, to me was the scientific frontier.

Early experimental evidence that take-all could be managed in continuous wheat monoculture was published in two PhD theses in Europe. One was the report of M. Gerlach in The Netherlands on the early development of take-all in new polders and its decline (Gerlach, 1968), and the other the work of Peter Shipton at Reading, UK, on soil assays to predict fields undergoing, or already into, take-all decline (Shipton, 1972). I met Shipton at the First International Congress of Plant Pathology held in London in 1968 and discussed with him the possibility that the virgin arid lands brought into wheat production with irrigation in eastern Washington might offer special circumstances to better understand take-all decline, similar to the virgin polder soils studied by Gerlach (1968) in The Netherlands. Shipton received a NATO Postdoctoral Fellowship for 2 years and joined me in Pullman in 1969.

We immediately began testing the applicability of Shipton’s soil assay for take-all suppression associated with putative take-all decline. This assay measured the severity of take-all on seedlings grown in pots filled with test soil (from wheat-monoculture fields) amended with a standardized amount of inoculum of

G. graminis var. *tritici* that we produced on an artificial food base. After nearly a year of ambiguous and inconsistent results, we decided to see whether it would be possible to transfer take-all suppression from a field in long-term wheat monoculture to a field with no known history of wheat or take-all. This idea did not arise in a vacuum. First, Menzies (1959), working with common scab of potato in the arid irrigated lands of eastern Washington, had reported suppression of common scab of potato with the addition of 10% scab-suppressive soil mixed into a scab-conducive soil. Second, we realized that with Shipton's soil assay the results were greatly influenced, if not dominated, by variations in soil type. We needed a common soil type as our rooting medium and to this we could add a standard amount of test soil (e.g. 1 or 10% w/w) as well as inoculum of the pathogen. Third, Baker, with all his experience in biological control, convinced me that our hypothesis was worth testing.

We selected six soils from eastern Washington fields, three with a history of wheat and three corresponding non-cropped sites near the respective three wheat fields (later described by Shipton *et al.*, 1973). The three fields cropped to wheat were: (i) (near Pullman) in a traditional wheat/pea rotation, with wheat every second or third year; (ii) (near Lind) in a traditional fallow/winter wheat rotation, with wheat every other year; and (iii) (near Quincy) in its 12th year of continuous wheat monoculture. The corresponding non-cropped sites were: (i) undisturbed grassland (near the Pullman field); and (ii) undisturbed native sagebrush-dominated vegetation (near the Lind and Quincy fields). Soil from the top 15 cm at each site was hauled over the Cascade Mountains to the Washington State University Research and Extension Center at Puyallup, Washington, where each was tested for its ability to initiate take-all decline. As a precaution against naturally occurring *Gaeumannomyces graminis* var. *avenae*, a related fungus affecting turf, the experimental site was fumigated with methyl bromide under clear plastic tarp at about 4.4 kg/m². Two days later, the tarp was removed and the six soils sprinkled uniformly and respectively over the surface of plots approximately 1.3 m × 3.0 m, with each soil treatment replicated four times. The two control plots included an equivalent rate of native soil and no soil (fumigated only). The entire site was then rotovated in the long direction of the plots and to a depth of 15 cm, starting with the no-soil control, followed by the native-soil control, then the non-crop (virgin) soil treatments, and finally the cropped soil treatments. The site was then planted to high-quality seed of winter wheat mixed in the drill box with oat grains colonized by *G. graminis* var. *tritici* (to assure the occurrence of take-all in the first season).

Arbitrarily deciding how much oat-grain inoculum to add and taking into account that the soil was fumigated, we overdosed and take-all was uniformly devastating that first year (1969/70 crop year). The site was planted a second time (1970/71 crop year) with wheat only, depending entirely on inoculum from the diseased crop from the 1969/70 crop year. In that second year, the effect of 'starter' soil from the 12-year wheat monoculture field near Quincy was spectacular. To the very border of each of the four replicate plots amended with this one soil, at the tillering stage the roots were still white, whereas in all the other treatments they were classic black from take-all (Baker and Cook, 1974; Fig. 44.1). The amount of 'starter' soil mixed to 15-cm depth amounted to only about 0.5% by weight. With the third sowing (1971/72 crop year), again depending entirely on inoculum



Fig. 44.1. Representative wheat plants from a field trial in the second year of wheat monoculture showing severe take-all (left and centre) or no apparent disease because of take-all decline (right) in response to the introduction of 'starter' soil (0.5% w/w, rotovated to 15 cm depth) 2 years earlier from a field in the 12th year of continuous wheat monoculture near Quincy, WA (right). Plots not yet into take-all decline (represented by plants on left and centre) were amended with the same amount of soil from a non-cropped (virgin) site adjacent to the 12-year wheat monoculture field (centre) or no soil (control, left). Take-all was uniformly severe in the first wheat crop and take-all decline occurred uniformly in the third wheat crop, regardless of the one-time initial soil amendment (Baker and Cook, 1974).

from diseased roots and stem bases of the previous crop(s), the wheat was uniformly healthy regardless of the treatment. Take-all decline had occurred throughout the test site.

I reached four conclusions from this one experiment.

- Something in soil from the field in continuous wheat monoculture was suppressive to take-all, it was transferable and it could multiply.
- The results of this experiment justified a full-scale project to explain them.
- There was no need to repeat this 3-year field experiment, since even if the performance of the soil from the Quincy field could not be confirmed that would not negate the results of this one experiment.
- Never again would I doubt whether take-all decline is real.

From Field Plots to Glasshouse Pots

With the Puyallup field tests underway, we began to refine Shipton's assay for specific suppression to take-all. We diluted test soils with a fumigated soil (our

standardized rooting medium) and amended this mixture with a standardized amount of ground oat grains colonized by the pathogen. This procedure was patterned after that described by Menzies (1959) and our field test for transfer of suppressiveness (Shipton *et al.*, 1973). Working with one part test soil blended with 99 parts stock fumigated soil, we confirmed in the glasshouse that the disease-suppressive factor was transferable. We also showed that the transferable factor was eliminated by steam-air pasteurization at 55°C, confirming the findings of Gerlach (1968) for the factor he associated with take-all decline in the Dutch polders.

However, it was not until 1973 when I was in Adelaide, Australia, working with A.D. Rovira that this pot assay system was perfected. The end of the 3-week incubation period of my first attempt with the assay in Adelaide coincided with the time set for me to give a seminar to the CSIRO Division of Soils faculty. I lined up pots representative of the treatments on a table next to the podium and hid them behind a large sheet of brown paper. After discussing the results of the Puyallup field experiment, and how this field test led to development of a pot test as a measure of suppressiveness, I ceremoniously removed the brown paper to reveal the row of pots, showing the tall plants in response to soil from the Quincy long-term wheat monoculture field and short (stunted) plants in response to the non-cropped virgin Quincy soil (Cook and Rovira, 1976; Fig. 44.2). I explained that each pot contained only 1% of the test soil, about a tablespoon, and the rest was the same

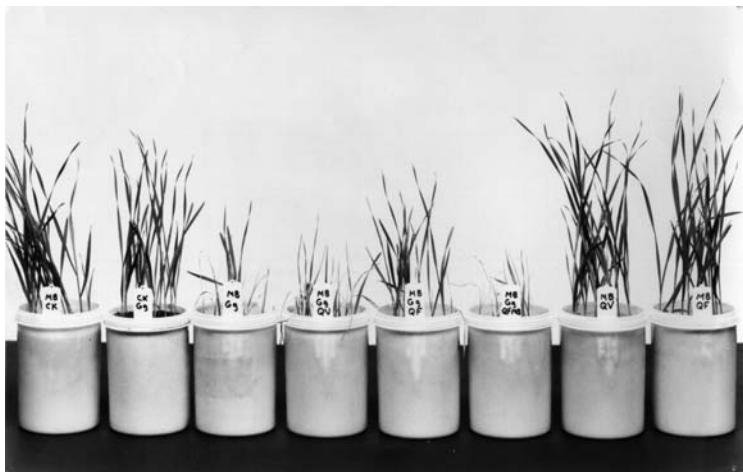


Fig. 44.2. Pot test showing the suppressiveness to *Gaeumannomyces graminis* var. *tritici* (Gg) of one part (by weight) soil from the continuous wheat-monoculture field near Quincy, WA (QF) mixed with 99 parts of a stock soil fumigated with methyl bromide (MB) (MB/Gg/QF), and the absence of suppressiveness in the stock fumigated soil amended with the pathogen only (MB/Gg), amended with one part soil from a non-cropped (virgin) site adjacent to the Quincy monoculture wheat field soil plus the pathogen (MB/Gg/QV), or amended with one part QF soil treated with methyl bromide plus the pathogen (MB/Gg/QF/MB). Controls include fumigated soil only (MB), natural soil with the pathogen only (CK Gg), fumigated soil amended with 1% of the QV soil (MB/QV) and the fumigated soil amended with 1% of the QF soil (MB/QF) (Cook and Rovira, 1976).

locally available stock fumigated soil used as the rooting medium. I then announced that I had come to work with the world's leading rhizosphere microbiologist to answer the question: what is in that tablespoon of soil?

Fast Forward to 2005

After more than 30 years of research, multiple PhD theses and postdoctoral projects and base support provided by ARS plus multiple grants from the USDA's National Research Initiative Competitive Grants Program starting in 1978, all evidence now indicates that take-all decline is a natural biological control caused by a specific genotype or select few genotypes of rhizosphere-inhabiting bacteria (rhizobacteria), taxonomically classified as *Pseudomonas fluorescens*. These bacteria are inhibitory to the take-all pathogen through production of the antibiotic 2,4-diacetylphoroglucinol (DAPG) (Weller *et al.*, 2002). While offering a milestone in rhizosphere microbiology that is arguably second only to the story of *Rhizobium* biology associated with nitrogen fixation, it is doubtful that the investment in research that has produced this remarkable story could or would have been justified without the unequivocal results of that early field trial laid out at Puyallup. Equally noteworthy, an estimated 2 million acres of crop land in the Inland Northwest is now cropped continuously or nearly continuously to cereals, using combinations of spring wheat, spring barley and winter wheat, with only minimal damage from take-all, presumably because of take-all decline. One can only imagine the millions of acres of wheat worldwide that benefited both health- and yield-wise because of the activity of this remarkable subpopulation or sub-subpopulation of DAPG-producing rhizobacteria.

A Brief Review of the Steps Leading to Today's Picture of Take-all Decline and Its Implications for Intensive Agriculture

By the time I left Australia in 1974, there was strong evidence to suggest that the factor suppressive to take-all in our pot test was: (i) operating in the wheat rhizosphere and not the bulk soil; and (ii) involved strains of fluorescent *Pseudomonas* species putatively inhibitory to the take-all pathogen (Cook and Rovira, 1976). One line of evidence came from the work of Smiley (1979), done prior to our work but published later, indicating that the disease suppression associated with ammonium nitrogen involved rhizoplane-inhabiting fluorescent pseudomonads inhibitory to the wheat take-all pathogen. D.M. Weller joined me in Pullman in January 1979, and isolated the now well-studied *P. fluorescens* strain 2-79 (Weller and Cook, 1983; Weller, 1988) from the rhizosphere of wheat in a pot test of soil from a plot on the Washington State University Research Unit, Lind, Washington, in the 10th year of continuous monoculture wheat. We thought at the time that this strain represented the population responsible for take-all decline as it provided significant suppression of take-all in the field plots (Weller and Cook, 1983; Fig. 44.3).

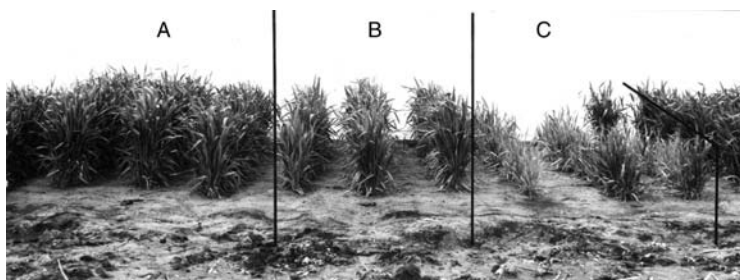


Fig. 44.3. Field test showing (B) the suppressiveness of *Pseudomonas fluorescens* strains 2-79 and 13-79 to take-all caused by *Gaeumannomyces graminis* var. *tritici* when applied as a mixture to the seeds of wheat with the pathogen introduced into the seed furrow, compared with (A) no seed-applied bacteria and no soil-applied pathogen (healthy control) and (C) pathogen added to the seed furrow but no seed-applied bacteria (diseased control) (Weller and Cook, 1983).

Weller was joined in 1985 by Linda Thomashow, and thus began their now-classic studies that led to the identification of the antibiotic phenazine-1-carboxylate (PCA) as the mechanisms of *in vitro* and *in vivo* inhibition of the take-all pathogen by strain 2-79 (reviewed in Weller, 1988; Weller *et al.*, 2002). Using Tn-5 mutagenesis, a plasmid that inserts itself randomly into genomic DNA, they generated mutant strains of 2-79 that lost ability to produce PCA and also became ineffective in disease suppression. Genetic complementation of the mutant restored PCA production as well as disease suppression in the rhizosphere. These results, together with quantitative documentation of PCA production in the rhizosphere of wheat inoculated with the wild type and complemented mutant, provided the first evidence, after decades of debate, that antibiotics are produced in soil and they play a role in the ecology of the producing microorganism.

Like so many culturable soil bacteria, the antibiotics produced by fluorescent *Pseudomonas* species has been described and structures worked out by microbiologists and organic chemists years earlier. They include pyrolectein, pyrrolnitrin, 2,4 diacetylphloroglucinol (DAPG) and the family of phenazines, each highly conserved worldwide within subpopulations of this large and diverse genus *Pseudomonas* (Weller *et al.*, 2002; Hass and Defago, 2005). With the basic chemistry worked out, an international effort by investigators interested in exploitation of these bacteria for biological control took this area of science to the next level by cloning and sequencing the genes involved in the biosynthesis of each of these antibiotics (Hass and Defago, 2005). Linda Thomashow led the effort that identified and characterized, respectively, a seven-gene locus for biosynthesis of PCA by strain 2-79 and a five-gene cluster for biosynthesis of DAPG in a strain of *P. fluorescens* Q2-87 isolated from the rhizosphere of wheat grown in the Quincy long-term wheat monoculture soil (Weller *et al.*, 2002). The use of genetic probes and primers specific for genes in the PCA and DAPG biosynthetic loci, along with colony hybridization and PCR, allowed quantification of PCA- and DAPG-producers in the rhizosphere of wheat grown, respectively, in suppressive and conducive soils and thus the testing of the hypothesis that one or both kinds

of antibiotics contribute to suppressiveness. These approaches showed that it is the subpopulation of *P. fluorescens* with ability to produce DAPG, not the phenazine-producing subpopulation as previously thought, that accounts for take-all decline under continuous wheat monoculture in Washington State and in The Netherlands (Weller *et al.*, 2002; De Souza *et al.*, 2003).

The threshold population of DAPG-producing strains of *P. fluorescens* required for take-all suppression is log 5 CFU/g root, and this amount was shown to occur naturally in the rhizosphere of wheat growing in soils that had undergone take-all decline (Weller *et al.*, 2002). Elimination of the DAPG producers eliminates disease suppression, whereas restoration of these bacteria to log 5 CFU/g root by mixing a small amount of take-all decline soil into conducive soil and planting wheat restores suppression. Further, the amount of DAPG produced in the rhizosphere of wheat is a constant 0.62 ng/10⁵CFU at populations of the DAPG-producing strain ranging between log 6 and log 7.

Polymorphisms in the *phlD* gene from the five-gene DAPG-biosynthetic operon, together with DNA fingerprinting, are now used worldwide to detect, quantify and characterize the distinct genotypes of DAPG-producing *P. fluorescens* in the rhizosphere of economically important crops (Weller *et al.*, 2002). At last count, 22 distinct genotypes of DAPG-producing *P. fluorescens* have been identified among the thousands of isolates obtained from rhizospheres (Landa *et al.*, 2005).

Of particular significance is the evidence that the genotype(s) dominating the population of DAPG producers in any give rhizosphere is modulated, in part, by the crop grown, length of monoculture and the geographic location of the field (Picard *et al.*, 2000; Landa *et al.*, 2005; McSpadden Gardner *et al.*, 2005). Among the DAPG-producing genotypes associated with continuous wheat monoculture and take-all decline, the D genotype has been the dominant strain in Washington take-all decline fields, whereas genotypes F and M were dominant in Dutch take-all decline fields (Weller *et al.*, 2002). Strain Q8r1-96 is of the D genotype (Weller *et al.*, 2002) and, like all genotype D isolates, is a highly aggressive colonist of the wheat rhizosphere, which no doubt contributes to its ability to efficiently suppress take-all in TAD fields under continuous wheat monoculture.

On the campus of North Dakota State University, Fargo, where wheat and flax were grown as monocultures in side-by-side plots for more than 100 years, populations of DAPG-producing pseudomonads exceeded the threshold log 5.0 CFU/g root in the rhizospheres of both wheat and flax grown in the soils. However, the genotypes that made up these two populations were very different. About 80% were of equal frequencies of genotypes F and J in soil where flax had been grown in monoculture, and 77% were genotype D in soil where wheat had been grown in monoculture. DAPG-producers were below the level of detection (10⁴ CFU/g root) on roots grown in soil from a third adjacent plot that had been in crop rotation (i.e. bean, maize, oat, soybean, sugarbeet, sunflower etc, or left fallow) for over a century (Landa *et al.*, 2005).

Similarly in a plot on the Washington State University, Northwest Research and Extension Center at Mount Vernon, Washington, DAPG-producers exceeded log 5.0 CFU/g root in the rhizosphere where peas had been grown in monoculture for the past 30 years and the soil was suppressive to *Fusarium wilt* caused by *Fusarium oxysporum* f. sp. *pisi*. Among the six DAPG-producing genotypes

identified in the rhizosphere of pea growing in this soil, D and P were dominant. Greenhouse studies showed further that D and P colonized the rhizospheres of wheat and pea, respectively, better than four other DAPG-producing genotypes (A, L, O and Q) also isolated from this pea-monoculture plot (Weller *et al.*, 2002). In Ohio, McSpadden Gardner *et al.* (2005) found the D genotype in the rhizosphere of maize, soybeans, or both crops in all 15 counties sampled, and it represented the most abundant of seven genotypes identified in total. On average, the D genotype was detected at populations exceeding log 3.4 CFU/g root on 77, 84 and 81% of maize plants sampled in years 2001, 2002 and 2003, respectively, and 78, 67 and 52% of soybean plants sampled during those three years, respectively.

In re-examining the early experiment on initiation of take-all decline in the Puyallup field plot with 'starter' soil transferred from a long-term wheat monoculture field (Shipton *et al.*, 1973; Baker and Cook, 1974), and the demonstration of take-all suppression with one part wheat monoculture soil mixed with 99 parts stock fumigated soil as the rooting medium (Cook and Rovira, 1976), it would seem nearly certain that the tiny amounts of soil that we used to transfer suppressiveness to the conducive soil contained genotype D isolates of DAPG-producing *P. fluorescens*.

A Brief Review of Concurrent Research with Biocontrol Pseudomonads

As in any advancement in science, the work on take-all decline is but part of a much larger and international effort in research on biological control with fluorescent pseudomonads over the past 30+ years. Here, and for a more proper perspective, I provide a very brief review of some of the concurrent, collaborative and supporting research with these ubiquitous and ecologically and agriculturally important rhizobacteria. The definitive and unambiguous studies continue to point to direct inhibition of soil-borne pathogens (antibiosis) as the primary reason for the obvious plant-growth enhancement by what have come to be known collectively as 'plant growth promoting rhizobacteria' (PGPR) (Kloepper *et al.*, 1980). However, the scope of this area of science continues to expand, with recent evidence that rhizobacteria can also suppress disease development through induction of systemic resistance (ISR) to plant pathogens, described as enhanced basal resistance in plants to their pathogens (Hass and Defago, 2005). Interestingly, the ISR depends on the jasmonic acid signalling pathway first shown to trigger a defence response in plants to herbivorous insects (Farmer *et al.*, 1992). In addition to these multiple mechanisms of plant growth promotion through biological control of root pathogens, genetic evidence is now also forthcoming for a direct role of rhizobacteria in promotion of root growth, including under gnotobiotic conditions (Wang *et al.*, 2006). For more comprehensive reviews, see Bakker (1989), Cook and Baker (1983), Hass and Defago (2005) and Weller *et al.* (2002).

One of the first lines of evidence that fluorescent *Pseudomonas* species had potential for biological control of soil-borne plant pathogens in the rhizosphere was from work done at the University of California, Berkeley, led by M.N. Schroth.

The seminal work from this group was their discovery that the enhanced growth of potatoes was associated with the production of siderophores proposed to inhibit pathogen growth through iron starvation (Kloepper *et al.*, 1980). Under Kloepper's leadership, an international PGPR workshop has been held somewhere in the world every 4 years since 1982. The 7th International Workshop on Plant Growth Promoting Rhizobacteria was held from 28 May to 2 June in The Netherlands.

Equally comprehensive work on the effectiveness of PGPR strains in biological control of pathogens in the potato rhizosphere has been done in The Netherlands. This work began with the report of increased growth of potatoes in response to treatment of potato seed pieces with fluorescent pseudomonads, with the greatest growth responses occurring in fields cropped every 3 years to potatoes (short rotation) compared with lesser growth responses in fields cropped every 6 years to potatoes (long rotations) (Schippers *et al.*, 1987). These results pointed clearly to a role of pathogen displacement or suppression in the rhizosphere since pathogen pressure (and hence response to pathogen control) would be highest in fields in short rotations (but not quite continuous potato monoculture). With no evidence that plant pathogens well known to be favoured by short rotations were inhibited by the introduced rhizobacteria, e.g. *Verticillium dahliae*, the hypothesis was advanced that the relatively poor performance of potatoes grown in short rotations is due to deleterious rhizobacteria enriched by frequent cropping to potatoes and that, in turn, are displaced or suppressed through iron starvation by the introduced PGPR (Shippers *et al.*, 1987; Bakker, 1989).

Parallel to the Berkeley and Dutch studies on growth promotion of potato, and about the time that D.M. Weller isolated strain 2-79, investigators working on cotton in Texas reported control of seedling blight caused by *Rhizoctonia solani* using one strain of *P. fluorescens* or its antibiotic, pyrolnitrin, and seedling blight caused by *Pythium ultimum* by a different strain of *P. fluorescens* or its antibiotic, pyroluteorin (reviewed in Cook and Baker, 1983). This research provides some of the first evidence, albeit circumstantial, for a role of antibiotic production in biological control of soil-borne plant pathogens by these bacteria. Whether the seed-applied bacteria colonized the rhizosphere of cotton or served only to protect the germinating seed against infection was not determined.

Similar to the take-all decline story, researchers in Switzerland led by G. Defago demonstrated a primary role of fluorescent pseudomonads in the suppression of black root rot of tobacco caused by *Thielaviopsis basicola* in a soil cropped 24 years to monoculture tobacco (Stutz *et al.*, 1986). Early work indicated that this example of disease suppression with monoculture of the host crop involved multiple mechanisms of iron starvation by production of siderophores and inhibition of the pathogen by production of hydrogen cyanide and antibiotics (reviewed in Hass and Defago, 2005). Subsequent work with *P. fluorescens* strain CHAO, obtained from the suppressive soil and now one of the premiere model strains for fundamental research, pointed to the importance of DAPG in this natural suppression (Keel *et al.*, 1992). *P. fluorescens* CHAO is member of the A genotype of DAPG-producing *P. fluorescens*.

Interestingly, the pyrolnitrin-producing strain *P. fluorescens* Pf-5, active against *R. solani*, also produces DAPG, is a member of the A genotype, and has become another model strain for fundamental studies. As another milestone, and an effort

led by Joyce Loper with ARS at Corvallis, Oregon and Linda Thomshow with ARS at Pullman, the genome of Pf-5 has been completely sequenced (Paulson *et al.*, 2005), revealing six secondary-metabolite gene clusters.

Still another in the growing list of DAPG-producing pseudomonads, and apparently the only known member to date of the K genotype (Weller *et al.*, 2002), has been shown in Ireland to suppress damping-off of sugarbeet and pea seedlings caused by *Pythium ultimum* (Fenton *et al.*, 1992), soft rot of potato caused by *Erwinia carotovora* (Cronin *et al.*, 1997a), and the potato cyst nematode, *Globodera rostochiensis* (Cronin *et al.*, 1997b).

Finally, and an interesting twist on the role of rhizobacteria in crop monoculture, soils not previously planted to apples are naturally suppressive to a fungal/oomycete complex of apple root pathogens but become conducive to these pathogens by the 3rd or 4th year after apples are grown in the soil, owing to a displacement of populations of microorganisms antagonistic to these pathogens by pseudomonads not inhibitory to these pathogens. This shift in microbial communities involving the concurrent enrichment in the inoculum density of root pathogens and populations of rhizobacteria that have no inhibitory activity to these pathogens accounts for the long-studied apple replant problem. However, planting wheat in old apple-orchard soil prior to replanting apples restores the population-inhibitory pseudomonads, in particular *Pseudomonas putida*, and coordinately the soil reverts from its disease-conducive to a disease-suppressive state and controls the apple replant disease (reviewed in Weller *et al.*, 2002).

What is Biological Control?

In his review of Baker's and my first book, Hirst (1974) wrote: 'The dilemma faced by the authors was how far to extend beyond the difficulty of defining biological control into the wider problems of managing microbial ecology to lessen plant diseases.' The definition put forward by Baker and Cook (1974) was 'Biological control is the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists.' Hirst (1974) considered this definition as 'so broad that it embraces all of microbial ecology', and preferred himself to restrict the term to control achieved by 'the manipulation of "third organisms" such as hyperparasites, antagonists, and competitors'.

Hirst's preference to exclude 'managing of microbial ecology to lessen plant diseases' from the concept of biological control of plant pathogens was incomprehensible to Baker and me at that time, and it is even more incomprehensible to me and probably most plant pathologists today. Restricting the concept in this way implies that unless the biological control agent is introduced or until one knows the resident agent, i.e. antagonist, responsible for the control, as in the case of take-all decline, it is not biological control. Yet Hirst's views were more nearly in line with 'conventional wisdom' at that time, possibly because of the many successes in biological control of insects and weeds with introduced agents. The late Ken Hagen, well known for his outstanding work and scientific leadership on

biological control of insects, tried to resolve what Hirst referred to as our 'dilemma' by suggesting to me in a personal conversation that most examples from plant pathology should be categorized as 'biological *methods* of control', as distinct from what entomologists considered 'biological control'. This struck me as splitting hairs and presumably would be as unnecessary as trying to distinguish between chemical control and chemical methods of control.

The definition offered in our first book and made more explicit in our second book (Cook and Baker, 1983) also included host plant resistance as biological control. Excluding host plant resistance from the concept of biological control always struck me as artificial if not political. Going back to Hirst's preference to restrict the concept to control achieved by 'the manipulation of "third organisms"', resistance induced in an otherwise susceptible genotype of the host by an avirulent or weakly virulent strain of the pathogen or PGPR strain would be included in the concept, but the same or similar mechanism(s) of resistance expressed in a resistant genotype of the host without the aid of the *third organism* would not be biological control. Whether resistance is manipulated through deployment of an organism or a gene, the control achieved is *biological* in nature and therefore logically should be included within the concept of biological control.

In a report released nearly 20 years ago, the US National Academy of Sciences defined biological control as 'The use of natural or modified organisms, genes, or gene products to reduce the effects of undesirable organisms (pests), and to favor desirable organisms such as crops, trees, animals, and beneficial insects and microorganisms' (NAS, 1987). This broad definition included the use of the crystalline protein of *Bacillus thuringiensis* as biological control whether delivered by the insect pathogen itself, a plant-associated microbe, e.g. endophyte or rhizobacterium genetically transformed to express the *Bt* gene, or as a gene expressed as transgenic resistance in the plant to the insect pest. Similarly, this NAS definition included as biological control of plant viruses both cross protection achieved by pre-emptive inoculation of the host with a weakly virulent strain of the virus or resistance achieved by expression of the coat-protein (or other gene) of the virus as a transgene in the host.

As scientists, we logically view problems and opportunities in science based on our own experiences and usually also by what we were taught by others within our own discipline. One can understand why those working on biological control of insect pests and weeds would focus entirely on regulating the population of the pest. By the classic definition of DeBach (1964), 'The use of pathogens, parasites and predators to regulate the population of a pest at a level lower than it would occur in the absence of these natural enemies', lowering the weed or insect pest population is the only acceptable outcome. Likewise, one can understand why those of us working on biological control of plant pathogens would focus on disease suppression, which, like the endophytic fungi antagonistic to herbivorous insects, may or may not involve reducing the population of the pathogen or pest. The focus in biological control of plant pathogens has been on protecting the health of the host, often with no reliable information on what happens to the population of the pathogen.

Whether or not one agrees with Hirst's preference to limit the concept to 'the manipulation of "third organisms"', scientists and policy makers today can justifiably ask: 'After 30+ years of research and investments of millions in research

dollars, where are the commercial products?' Indeed, the standard for documentation that a strain fits the definition of PGPR (Kloepper *et al.*, 1980), and now applied by investigators worldwide, is to document an improved stand, increased growth and/or higher yield in response to the strain or strains introduced on or with the seed or other planting material at planting. Our work on take-all at Pullman, and similar work in Australia and China, has involved extensive and intensive field testing of these strains over a 20-year period, starting with the PCA-producing 2-79 (Weller and Cook, 1983) and then with the DAPG-producing D-genotype Q8r1-96 (Cook *et al.*, 2002). However, with few if any exceptions, the increased growth and yield responses have been too variable or inconsistent to meet the standards set for commercialization (Weller, 1988; Bakker, 1989). I have argued unsuccessfully that the standards are unrealistically high (Cook, 1993), and that development and use of microbial biocontrol products should be modelled after the development and release of plant varieties rather than modelled after pesticides. Obviously no company is going to invest in the cost of obtaining regulatory approval under standards set for pesticides, especially if the microbial agent will be used for biological control of only one disease on one crop. Yet experiment stations and seed companies have released thousands of crop varieties based on a single superior trait or slightly improved agronomic performance.

PGPR strains introduced with the planting material, or by other means, should eventually expand from their current minor use to become part of mainstream agriculture, including as strains genetically engineered to express more than one antibiotic (Fenton *et al.*, 1992; Blouin Bankhead *et al.*, 2004). However, for now we are left with the approach that Hirst (1974) excluded from the concept of biological control, i.e. 'managing of microbial ecology to lessen plant disease', and what Baker and Cook (1974) included in the concept, which is '...reduction of ... disease-producing activities of a pathogen ... by one or more organisms, accomplished naturally ... through manipulation of the ... host. ...' Even more intriguing than the millions of acres of intensive cereals that benefit health- and yield-wise year after year because of the activity of DAPG- and other antibiotic-producing rhizobacteria, it would be interesting to know the area of crops, turf or plants in natural ecosystems more generally that are healthier because of the antibiotic-producing rhizobacteria that team up with roots of their host to provide biological control of the soil-borne pathogens of that host.

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45

The Biocontrol Network: a Canadian Example of the Importance of Networking

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Overview: The Canadian government invested over CA\$6 million during 2001–2006 in a primarily academic research network aimed at developing biologically based pest management methodologies for contained ecosystems (greenhouses, tree nurseries and managed forest stands). The Biocontrol Network achieved a number of scientific successes over its brief span of 5 years. Most notably, it established an environmentally sensitive *culture of biological control* among researchers and students across Canada, which could serve as a model for the much more ambitious application of its principles to the open systems of crops, forests and the larger environment.

Introduction

Canada has an export-driven economy, in which agricultural and forest products play a central role (over CA\$66 billion in 2005). Many Canadians demand products grown free of pesticides, now increasingly de-registered in Canada and abroad because of adverse health effects. Add to this the deleterious environmental effects of pesticides, and it is clear that an alternative means of plant protection is needed.

Canadians have a long history of developing living organism substitutes for the chemical pesticides that protect their crops and forests. Several Canadian agencies work in biological control along with university programmes in integrated pest management. However, the effort is fragmented despite recent efforts to integrate R&D resources. Canada's sparse record in producing commercial biocontrol products compared to the USA and Europe reflects a lack of planning in carrying research on biocontrol agents, pests and commodities forward to formulation, commercial production and product marketing. The most significant barriers to developing a Canadian biocontrol industry have been the small

market size and, until very recently, an unfavourable regulatory environment. As Mark Winston of Simon Fraser University pointed out, 'this research has been largely uncoordinated, inadequately prioritized and too little effort has been made to translate the research into practical applications' (Winston, 1997).

This background set the stage for the creation of the Biocontrol Network, the first national organization of its kind to confront the broad-based challenges of developing biocontrol products for the food and forestry industries.

History of the Biocontrol Network Initiative

In August 1997, at the Society for Invertebrate Pathology annual meeting in Banff (Alberta), researchers from academia and government working in plant protection recognized the need for a comprehensive effort to reduce pesticide use in Canada through biocontrol. Canadians already possessed much of the needed expertise to protect the country's agriculture and forests with biological tools and pest management strategies. From this came the Biocontrol Network initiative, which aimed to connect the scientific community to the beneficiaries of the research – growers, foresters, biocontrol companies, extension experts, regulators and environmental/consumer protection organizations. The aim was to create a comprehensive R&D programme focused on biological control as an alternative strategy to chemical pesticides.

The plan called for a collaborative, Canada-wide network among biocontrol laboratories, in which the research of one discipline would benefit from advances in other disciplines. Administrative structures would address the bottlenecks in scientific understanding and commercial development, and ensure that the biocontrol agents developed were integrated into existing pest control strategies. Further, there would be a plan to deliver these pest control solutions to Canadian farmers and foresters. Consultations held by the organizers (J.-L. Schwartz and R. Laprade, Université de Montréal, Quebec) with Canadian groups over the next year identified the needs of stakeholders, and this became the principal driver of the research programme. With significant support from across Canada, they developed a proposal involving over 150 investigators from universities and public research institutions. They organized the research initially by expertise group (i.e. bacteria, viruses, fungi, macro-invertebrates, etc.), but then to encourage interactions among disciplines, re-shaped and re-focused it in early 1999 into three ecologically based themes centred on commodities and a fourth on interactions among plants, pests, pathogens and predators at population, environmental and microscopic (molecular biology and physiology) levels (Fig. 45.1). This was submitted to the Canadian government's Networks of Centres of Excellence programme competition but was not selected in light of other pressing national priorities.

The organizers then further refined their programme, and with about one-third of the original scientists, submitted another proposal to the Research Network Grants Program of the Natural Sciences and Engineering Research Council of Canada (NSERC). The objectives of this programme (see NSERC website) were almost ideally suited to the re-designed network, which confined

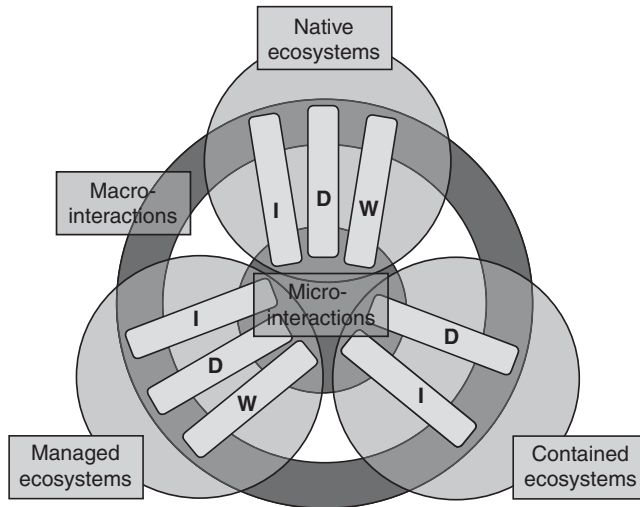


Fig. 45.1. A comprehensive and integrated view of networked biocontrol research. Network research addresses issues of biologically based pest management (I: insects, W: weeds, D: diseases) in three major ecosystems (native, e.g. boreal forest; managed, e.g. open field crops; contained, e.g. greenhouses), with particular emphasis on interactions (dark ring: macro-interactions e.g. inter- and intra-guild dynamics, and dark circle: micro-interactions e.g. physiological and molecular relationships). Experts of various disciplines work together on specific programmes, sharing experience, trainees and resources.

its research to pest problems in greenhouses, tree nurseries and managed forest stands. The new programme positioned the network at the initial stages of the natural development cycle of biocontrol products where laboratory ‘models’ are developed and scaled up in contained systems like greenhouses and tree nurseries, and then applied to larger, open systems (farms, forests and the wider environment). The Biocontrol Network received NSERC funding of CA\$1.3 million annually over 5 years and began operations in 2001 with additional support from a large group of public and private partners. Network researchers came from 16 Canadian universities, one college, 16 government research agencies and two non-profit research organizations (including CABI in Switzerland). The Network was managed out of the Université de Montréal in Quebec.

The Biocontrol Network

Serving industry

The Network’s mission was to reduce the use of chemical pesticides in Canada’s greenhouse and tree nursery industries using biocontrol methods. These industries are worth billions of dollars annually and are growing steadily in importance. In 2003, the Canadian greenhouse industry had revenues over CA\$2.5 billion and

employed 43,000 people for a total payroll of CA\$516 million (see Agriculture and Agri-Food Canada, and Statistics Canada websites). The Canadian tree nursery industry is a third the size of the greenhouse industry (15,000 employees with a payroll around CA\$200 million) (see Natural Resources Canada – Canadian Forest Service website) but its economic impact rises dramatically in light of it being the crucial base of the forest renewal programmes that underpin a cornerstone of the Canadian economy, the CA\$60–70 billion forest products industry.

Nature of the problem

Both greenhouses and tree nurseries are afflicted by pests and diseases. To address the problem and maintain premium-quality products, there is a continued need for improved or new biocontrol agents, and knowledge of the interactions between pests, diseases, plants and biocontrol agents. Although chemicals are not likely to be completely excluded as pest management controls, worried regulatory agencies are increasingly de-registering chemicals because of their toxicity. Added to the problem is the rapidly growing resistance of pests to chemicals, and the fact that chemical residues on crops and their distribution in the environment constitutes a growing health concern.

Diversified researchers and programmes

There were about 60 researchers in the Network from many disciplines. Biologists, mycologists, virologists, bacteriologists and physiologists examined the attack strategies of insects and disease pathogens on plants. Biochemists, biophysicists and cell physiologists investigated the mode of action of microbial agents and developed screening assays for pathogens active against pests. Ecologists studied the complexities of manipulating entire biological systems to control pest populations. Economists tracked the impact of biocontrol on the Canadian economy and alternative production routes for biocontrol agents. Social scientists confronted ethical issues, did risk assessments and evaluated public perceptions of biocontrol. The Network harnessed the talents and energy of this diverse group in a coordinated, focused way.

Network Research Programme

The aim was to understand the biological mechanisms and species interactions in greenhouses and tree nurseries in order to predict outcomes and anticipate future problems. There were three broad research themes: greenhouses, tree nurseries and managed forest stands, and innovation tools for discovery and testing.

Theme 1 sought to reduce the damage to greenhouse crops by insects and mites, and protect roots and leaves from disease. Horticultural and ornamental crops are often protected by one or more species of predatory, parasitic or

pathogenic organisms that establish themselves in crops and eventually control pests, allowing growers to reduce or eliminate pesticide use. But new pests continue to appear, raising the need to develop novel biocontrol solutions. To do this, knowledge is needed of the dynamics of pest communities and the behaviour of biocontrol agents operating together, as well as methods of manipulating these living communities. There were three greenhouse programmes: (i) plant pest–natural enemy interactions and biocontrol applications; (ii) improved microbial agents for management of insect pests; and (iii) management of rhizosphere (roots) and phyllosphere (leaves) ecology in greenhouse-grown crops.

Theme 2 focused on tree nurseries and managed forest stands. Tree seedling production and planting are major Canadian businesses, with 600–700 million seedlings (mostly conifers) produced annually in nurseries for reforestation programmes (see Natural Resources Canada – Canadian Forest Service website). Seedlings are produced under growth-intensive conditions in nurseries and are attacked by root diseases (rot and damping-off pathogenic fungi) and foliar pests (balsam fir and redheaded pine sawflies). There were three areas of research: (i) the discovery and development of biological control agents against diseases; (ii) research into nucleopolyhedroviruses (NPV) as biocontrol agents in tree nursery and managed tree stand models; and (iii) biocontrol of insect pests in Christmas tree plantations to understand the cyclical insect pests and their natural enemies.

Theme 3 aimed to develop new technologies based on DNA microarrays, as well as screening models and assays. These two programmes centred on: (i) microarray analysis of cellular responses as they relate to research into molecular and cell biology interactions among plants, pests and biocontrol agents; and (ii) development of cell and tissue models and assays for studying the mechanism of action of novel biocontrol agents and the screening of pathogens using advanced optical and electrophysiological techniques. Both investigative tools had applications across the research programmes of the Network.

Translating Network research into products for pest management programmes

The Network had a seven-stage research and development process, which allowed pest management tools at different development stages to be included in the Network programme and promoted information exchange between researchers working in different systems but addressing similar problems. The design of the seven-stage process was as follows: (i) *project initiation*: identify user needs, preliminary economic and environmental assessment, project proposal; (ii) *ecological studies*: life history of pest and existing control mechanisms, pre-release (baseline) levels of pest and biocontrol agent(s) monitoring; post-release of product efficacy evaluation, environmental impact studies; (iii) *screening*: potential biocontrol agents identification, lead candidate(s) selection; (iv) *product development – production*: small-scale production, formulation research or genetic modification, scale up for release and commercialization; (v) *product*

development – efficacy: greenhouse and field trials (involvement of growers); (vi) *product development – environmental impact*: preliminary and advanced studies (e.g. ecological studies), complete registration data package; and (vii) *marketing, training and education*: ongoing assessment of economic feasibility, market research studies, users and general public training and education. Several stages were found in programme activities of the Network (below).

The Network – a Science Success

There were many success stories in the work carried out by Network scientists over the years. Several are described in other chapters of this book. They include: the development of a new insect predator for use in greenhouses (see Gillespie *et al.*, Chapter 14 this volume); the discovery of resistance to *Bacillus thuringiensis* (Bt) by the cabbage looper (see Janmaat, Chapter 19 this volume); the registration and commercialization of Sporodex (*Pseudozyma flocculosa*), a unique biofungicide against powdery mildew (see Jarvis *et al.*, Chapter 25 this volume); the development and registration of Chontrol (*Chondrostereum purpureum*), a re-sprouting inhibitor (see Hintz, Chapter 31 and de la Bastide and Hintz, Chapter 32 this volume); and the registration of Abietiv, a baculovirus-based agent against the balsam fir sawfly (see Lucarotti *et al.*, Chapter 39 this volume).

Other Network successes

Prestop effective against Pythium and Fusarium

Research demonstrated the efficacy of Prestop (*Gliocladium catenulatum*), a biofungicide effective against *Pythium* and *Fusarium* diseases in Canadian greenhouses (Bélanger, 2007). This led the growers to allocate top priority to Prestop registration in the first (2003) minor-use prioritization exercise of Agriculture and Agri-Food Canada's new Pest Management Centre. This work was a partnership with Verdera Oy of Finland. Further Network research on biopesticide development and registration of this and other products was supported by a substantial grant from Quebec's Department of Environment.

Studies of parasitoids to control lygus bugs

Lygus bugs are major agricultural pests. The Network partnered with CABI (Delémont, Switzerland) to develop a classical biocontrol strategy against the bugs. This will have a major economic impact, although it is by nature non-commercial. Normally, the work involves long, complex and costly host-range studies to ascertain the possible negative effects of introducing a biocontrol agent against *Lygus* into a new environment. To speed things up and increase efficiency, Network researchers developed a one-step molecular tool to detect and identify key European parasitoids recognized as potential classical agents against *Lygus* in Canada (Gariépy *et al.*, 2005).

Genomics of healthy rhizospheres

A Network genomics profiling of nursery rhizospheres may be scaled up for high throughput screening of biological control agents. It turned out that there were gene differences between rhizospheres associated with healthy and diseased black spruce seedlings. Further, these differences could be used to target specific organisms as potential biological control agents. Small-scale DNA sequencing of specific antibiotic-synthesis genes in soil bacteria and Actinomycetes revealed genetic variations among species. This work, the first culture-independent study of microbial diversity in conifer nurseries, confirmed that specific mutations are associated with different levels of antibiosis (Fillion *et al.*, 2004).

Hybrid parasitoid against spruce sawfly

Network researchers studying parasitoids that prey on yellow-headed spruce sawfly or pine false webworm partnered with Beneficial Insectary of Redding, California. The work generated considerable interest among forest management companies. Because collecting native parasitoids from the sawfly is difficult, the work switched to a European parasitoid species with a more restricted host range. The crossing of European and native species led fortuitously to fertile hybrids with an extended host range. This enabled mass production and the promise of using the parasitoid in integrated pest management of forests.

Insects under stress

DNA microarrays indicated that apoptosis (suicide) genes were induced in eastern spruce budworm midguts in response to sublethal levels of Cry1A toxin from *Bt*. A suppressive, subtractive hybridization procedure was applied to dissected midguts of the budworm exposed to sublethal levels of the toxin (Meunier *et al.*, 2006). In another project, an oligonucleotide-based DNA microarray (*cryArray*) was designed and validated for the identification of the *cry* gene content of *Bt* strains. Since one *cryArray* hybridization could replace over 30 individual PCR reactions, these microarrays should become excellent tools for fast screening new *Bt* isolates with potential insecticidal activity (Letowski *et al.*, 2005). A microarray was also produced and validated with all the potential reading frames of TnSNPV, a newly discovered baculovirus. The global analysis of viral gene expression data obtained should have considerable impact in characterizing successful versus abortive infection in insects.

The insect gut microenvironment determines B. thuringiensis toxin activity

An important breakthrough was made possible by using an electrophysiological approach on the isolated midgut. It creates gut conditions (ionic force, pH, proteases, etc.) which can be manipulated, and qualifies as the *in vitro* model closest to the *in vivo* situation (Fortier *et al.*, 2005). Site-directed mutagenesis and two optical assays based on scattered light intensity and on fluorescence quenching were used to study, under various pH and ionic conditions, the importance of crucial regions of *Bt* molecules and proteases in pore formation (Kirouac *et al.*, 2006). It was found that toxin-induced increased gut apical membrane permeability to amino acids and divalent ions allowed the ruling out of alternative interpretations for the cellular mode of action of the toxins, confirming that pore formation

was the key phenomenon of their activity, and that the pores are freely permeable to divalent cations. These biophysical approaches constitute excellent prototypes for the development of high-throughput screening tools for novel biocontrol agents.

Beyond the Network laboratories

Networking

The Network has served as a single point of entry into a disparate field, bringing together the multidisciplinary expertise of researchers across the country and integrating them into research programmes aimed at alternative solutions to chemical pesticides. The Network linked up the relevant stakeholders in the field: researchers, growers, biopesticide and seed companies, government regulators and environmental monitoring groups.

Two-thirds of the Network's scientists collaborated with four or more other members in at least one research programme. More than half the published Network papers in 2004 were co-authored by two or more Network scientists, and this jumped to over 70% in 2005. Networking was nurtured by regular annual meetings and several regional meetings across Canada. Many Network scientists participated in the *Réseau québécois de recherche en phytoprotection* (Plant Protection Research Network), a Quebec offshoot of the Network. The Network co-organized an international symposium on 'Pesticides and Health' in Montreal in 2003; there was a joint meeting of the International Organization for Biocontrol of Noxious Animals and Plants (IOBC)–Nearctic Regional Section (NRS) and the Biocontrol Network in Magog–Orford, Quebec, in 2005, which included a special symposium on 'Trophic and Guild Interactions in Biological Control' and an IOBC Global 50th anniversary session on 'Biological Control to Support Biodiversity'. The same year, the Network organized the 6th Pacific Rim Conference on the Biotechnology of *Bacillus thuringiensis* and its Environmental Impact in Victoria, British Columbia. It was also invited to organize, in 2007, the 40th Annual Meeting of the Society for Invertebrate Pathology (SIP) in Quebec City and the Joint IOBC–Arthropod Mass Rearing and Quality Control–Association of Natural Biocontrol Producers–International Biocontrol Manufacturers Association meeting in Montreal.

Training

The Network's multidisciplinary research programme provided a unique environment for students and other trainees. Close to 180 students, postdoctoral fellows and technical assistants were trained, half of them being co-supervised by Network researchers. Five international competitions were run for the Network's prestigious postdoctoral fellowships and graduate scholarships. Two summer schools were organized, one in Windsor, Ontario, in 2004, and the other in Magog–Orford in 2005, following the joint IOBC/Biocontrol Network meeting, attracting over 130 participants. The Network awarded student grants and cash prizes to support travel and to recognize the best presentations at various conferences in Canada and abroad, for example at the meetings of the

Entomological Society of Canada, the Canadian Phytopathological Society, the SIP and several IOBC meetings.

Education and communications

With its host institution, the Université de Montréal, the Network created a new Canadian Research Chair in Biocontrol at a senior level, with full funding from the Canada Foundation for Innovation. The first recipient of this prestigious position was Dr Jacques Brodeur, a former member of the IOBC–NRS Board of Directors and a Network programme co-leader. A website was set up (see Biocontrol Network's website) with information on research programmes, participants, meetings and other activities. The site linked to other biocontrol-related websites worldwide and provided the first comprehensive list of biocontrol products registered in Canada. With the World Wildlife Fund of Canada and Agriculture and Agri-Food Canada, the Network published the quarterly *Biocontrol Files* (in English) and *Dossiers Biocontrôle* (in French), which was e-mailed to over 1200 individuals and posted on the Network's website. The Network sponsored the present book, a major contribution to the field with a number of contributions of Network researchers. In addition, a book on trophic and guild interactions was published (Brodeur and Boivin, 2006) and another on *Bt* biotechnology and its impact (Côté *et al.*, 2007) is slated for publication in early 2007. The Network had a broader public communications strategy based on the outcomes of an extensive national survey which gauged the Canadian public's perceptions of biological control. Two important studies were commissioned by the Network: (i) evaluation of the social and economic impacts of biocontrol R&D in Canada; and (ii) evaluation of barriers to commercial application of biocontrol products in Canada and alternative approaches to overcome these difficulties.

Synergies

Almost 50% of the Network's scientists had dual roles, doing research in government laboratories while holding university teaching positions. This link-up brought a stronger, practical focus to academia, sensitizing it to the product-oriented considerations of industry. In turn, industry and government laboratories were refreshed by academia's creative atmosphere and focus on fundamental research. The Network was a rich training ground for students, opening up a variety of career choice options. In fact, a 'biocontrol culture' was created, arguably the Network's most important added value to Canadian science.

For industry, the Network provided one-stop shopping for projects with commercial potential, expert advice, and a source of skilled staff. For regulatory agencies, it did the research to establish, improve and interpret regulations governing biological pest management. It provided the structure that permitted field efficacy and registration studies in multiple locations across the country, ensuring maximum relevance and acceptance. It played a vital educational and consultancy role, especially concerning registration requirements. The Network helped by performing basic research into the parameters governing product efficacy (required for new pesticides in Canada) by providing multi-level approaches and educating users on the new approaches. For farmers, foresters, crop protection specialists, industry and other end-users, the Network provided information and

training in implementation of ecologically based pest management programmes. For the Canadian public, it acted as a repository of clear, scientifically based facts on pest control with live organisms and provided the information needed to make informed decisions on public policy. For foreign biocontrol and integrated pest management (IPM) groups, the Network was Canada's single point of reference, facilitating global approaches to research, practical applications and regulatory issues. The latter enhanced Canada's international image of a country sensitive to resource sustainability, human health and the environment.

A recipe for success

A number of factors came together over the Network's 5 years to make it a success. We established a clear vision of what the Network was to be, and defined our objectives and action plans. We wanted to create a network *culture* in laboratories across Canada, with researchers and trainees from disparate disciplines working on common goals. Early on, we focused narrowly on greenhouses and tree nurseries, the first step in a natural process towards open systems (crops, forests and the larger environment).

Strong, flexible leadership through an inclusive, collegial process was essential to make decisions on the Network's evolving shape and directions. We established a unity of purpose early by clearly defining what we meant by 'biocontrol', ensuring unambiguous communication among the scientists and stakeholders. We grouped our researchers under application themes rather than discipline, encouraging disciplines to work together in integrated programmes. This transformed our differences into a source of strength. And, critically, we used our money creatively to allow laboratories to hire personnel for Network projects and support networking centrally. We also maintained significant funding for valuable, unexpected projects (e.g. aquaponics or bioagent dispersion by bumblebees; see Kevan *et al.*, Chapter 35 this volume).

Our managerial priorities always reflected the Network's community thinking. Science quality was always foremost, training strongly promoted, networking enthusiastically supported, and the sharing of research knowledge (with growers, regulatory agencies, biocontrol industry) vigorously encouraged. We moved early to create a culture of broadly based decision making. Finally, we strongly proselytized biocontrol as an option for plant protection to a broad audience in as many venues as our resources allowed. In the end, we created a culture of like-minded researchers and students, all speaking with the same voice to advance our goals of developing biological alternatives to chemicals for protection in greenhouses and tree nurseries.

A Retrospective: the Lessons of the Biocontrol Network

Looking back, it is easy to trace the highlights in this Canadian story. Most important, the Network achieved a high level of research excellence in biocontrol of greenhouses, tree nurseries and managed forest stands. Its programmes were

multidisciplinary, with emphasis on synergism among investigator efforts. It provided exceptional training for a long list of trainees schooled in a framework of multidisciplinary training, co-supervision between laboratories and intensive summer school programmes. It delivered improved or new products and strategies for many partners, and its effort expanded with time beyond the laboratory to include economic, social, ethical and health issues. It became an active partner with regulators. Several communications and outreach tools were developed, and high visibility and international recognition were gained through strong representation at several scientific meetings worldwide and by organizing international events in Canada.

The clarity of hindsight also points to a key endeavour that the Network did not pursue as assiduously as it should have, and one that could have made a crucial difference to finding the financial support to ensure continuance: building stronger linkages with the Network's stakeholders and community of opinion leaders. From the outset the Network should have moved to identify and develop other avenues of support for its programmes, such as matching-funds arrangements, despite scarce resources in the biocontrol industry and growers groups. Much more should have been made of the Network's importance to ancillary groups with political clout such as environmental and consumer organizations. The Network should have developed an advocacy programme that promoted its value in the wider community, beyond science, to groups with broader concerns such as global warming and the coming changes in insect and disease distribution on the planet. The Network needed to 'get on the radar' of news media and others seeking informed opinion on issues surrounding pesticide use and climate change. Organizations like the Biocontrol Network will always need some level of public funding and should always have an eye on moving up the list of political priorities at provincial and federal levels of governance.

On the eve of publishing this manuscript, the Biocontrol Network has not yet received further funding and its future remains in doubt. If such support is forthcoming, however, the Network will undertake a number of changes in the future to accomplish its longer-range mission (Fig. 45.2):

- 1.** The research will be expanded beyond crop protection in greenhouses and tree nurseries to other larger, open ecosystems. The Network's environmentally friendly approach needs to be extended to field crops, native forests and urban recreational settings. This is a natural evolution, taking the lessons from controlled, contained systems to larger, open systems.
- 2.** Greater efforts will be made to connect Network programmes more closely to IPM research and development to increase their practical relevance.
- 3.** Network programmes will be developed to address direct threats to health and the environment from global climate change (e.g. insect-borne human diseases, plant pests moving northward).
- 4.** The Network's contingent of investigators will enlarge to include other disciplines such as economics, social sciences, public health, public policy, ethics and communications.
- 5.** Growers and foresters will be encouraged to play more active roles in advocating biocontrol approaches and convincing the public of the benefits of biocontrol.

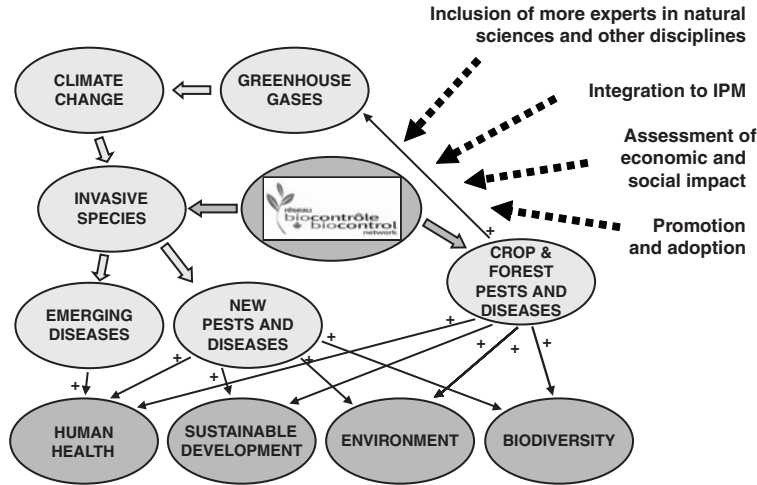


Fig. 45.2. Current context and future role of the Biocontrol Network. Fat arrows indicate the interconnections between global issues, the Network’s current role (crop protection) and its future role (addressing global issues). Fat, dashed arrows indicate planned additions to the current Network in terms of people and activities. Thin arrows with + signs indicate the positive impact that the Network’s current and future activities will have on global issues.

This will involve working with regulators, communicators, advocacy groups and political lobbyists to better understand the factors hampering the implementation of biocontrol. The Network effort should be as much about improving the public’s understanding of the process as about research and development.

6. The Network will place greater emphasis on measuring the economic advantages of biocontrol to its stakeholders, its longer-range positive effects on the environment and its general advancement of the larger ‘public good’.

Canada’s Biocontrol Network has, to date, succeeded in building the footings for a far larger and more ambitious initiative to capitalize on the promise of all its groundwork. No other organization existed anywhere in the world that both linked *and* funded a full spectrum of academic, government and commercial biocontrol researchers seeking and applying research results, and training the next generation of highly qualified personnel along the way. The Biocontrol Network’s pioneering spirit has been encouraged and supported for five exciting, productive years, and will surely evoke emulation.

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- Biocontrol Network: <http://www.biocontrol.ca>
- Natural Sciences and Engineering Research Council of Canada: <http://www.nserc-crsng.gc.ca>
- Natural Resources Canada – Canadian Forest Service: http://cfs.nrcan.gc.ca/sof/sof06/statistics_e.html#ForestHealthAndSilviculture
- Statistics Canada: <http://www40.statcan.ca/l01/cst01/agrc31a.htm>

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Index

- 2,4 diacetylphloroglucinol 406
Abelmoschus esculentus 278
Abies balsamea 353
Abutilon theophrastis 278
Acantholyda erythrocephala 360
acaricide 375, 377
Acer macrophyllum 288
Acer rubrum 288
acervuli 277
Aculus schechtendali 378
Acyrtosiphon pisum 42
Adalia bipunctata 45
additives 269, 281
Adelges tsugae 41
Adoxophyes orana 339
Aequorea victoria green fluorescent
 protein 332
aflatoxins 241
aflatoxin 241–253, 254–261
Agapta zoegana 72
Agelaius phoeniceus 93
Ageniaspis fuscicollis 15
Agistemus fleschneri 374
Agria mamillata 18
Aleiodes indiscretus 56
allelopathic control 225
allergenic 250
allergies
 insect 49, 80–91
 weed 80
Alnus rubra 284
Alnus rugosa 288
Alnus viridis ssp. *sinuata* 288
alpha-galactosidase 255
Althaea rosea 278
amber disease 162, 163
Amblyseius aerialis 30
Ambrosia artemisiifolia 80
Ambrosia trifida 84
Anagasta kuehniella 170
Anastatus disparis 56
antagonist 192, 226, 229, 230, 264
antagonistic 400
antibiosis 235, 408
antibiotic 230, 406
 metabolites 205
 -producing organisms 264
 -producing strains 263
Anticarsia gemmatilis 344
antifungal activity 230
antimicrobial
 compound 269
 peptides 266
Anystis baccarum 374
Apanteles glomeratus 21
Aphelinus 113
aphid 383
Aphidius 113
Aphidius ervi 47
Aphidius matricariae 121
Aphidoletes 113
Aphidoletes aphidimyza 46, 121, 139

- Aphis citricola* 42
Aphis glycines 42
Aphis gossypii 41, 42, 113, 383
Aphis nasturtii 43
Aphis spiraeicola 41, 42
Apis mellifera 172, 320, 360
Aporia crataegi 21
 apple 336
 ermine moth 13
 scab 234
 Arabidae 56
Arion ater 157
Artemisia vulgaris 82
Aschersonia 113
Asclepias syriaca 319
 Ascospores 220
 Asian citrus psyllid 41, 42
 aspen 288
Aspergillus flavus 241, 242, 254
Aspergillus niger 255
Aspergillus oryzae 255
Aspergillus parasiticus 245
 assay 205, 237, 403
Athelia bombacina 235
 atoxigenic 241
 aubergine 114
 Australian plague locust 316
- Bacillus anthracis* 174
Bacillus subtilis 149, 262
Bacillus thuringiensis 113, 149, 154,
 169–178, 179–184, 330
 resistance 179
Bacillus thuringiensis var. *israelensis* 122
Bacillus thuringiensis var. *kurstaki* 122,
 322
 Baculoviruses 336–343, 344–352,
 353–361, 362–373
 recombinant 362
Balaustium 16, 374
 balsam fir sawfly 353
 banker plants 122
 barley 399
 basidiospores 215, 285, 292
Beauveria bassiana 95, 122, 199,
 300–310, 312, 322
Bemisia tabaci 113
 benchmark 186
Beta vulgaris 278
Betula papyrifera 288
- big-leaf maple 288
 bioassays 173, 348, 364, 369
 Biocontrol Network 415
 biodiversity 96, 97
 biofilm 267
 biofungicide 231, 236
 bioherbicide 274, 291
 biological control
 conservation 374–382, 383–391,
 383–391, 386
 definition 2, 410
 in greenhouses 105–117, 118–127,
 128–135
 inundative 86
 multiple-species 70
 network 415–427
 biological invasions 83
 biopesticide 197
 microbial 160
 viral 336–343
 biosanitation 234, 238
 birds 256
 black cottonwood 285
 black vine weevil 137
 blackmargined aphid 41
Blepharipa pratensis 56
Blumeria graminis 228
 boll weevil 383
Bombus impatiens 320
Bombyx mori 169
Botrytis 262
Botrytis cinerea 113, 268, 320
Botrytis squamosa 239
 boundary layer 230
Brachiacantha ursina 45
Brachymeria intermedia 56
 Braconidae 56
Brassica napus 322
Brassica sp. 278
 broccoli 180
Bromus tectorum 77
 brown citrus aphid 41
 buckthorn aphid 43
 buckwheat 394
 bumblebees 113, 123, 225, 320
- cabbage loopers 179
 cacao 210
 cadavers
 entomophthoralean 54, 57

- gypsy moth 57, 60, 61
- insect 137, 369–370
- slug 155, 157
- Caenorhabditis elegans* 172
- calcium chloride 268
- Calosoma sycophanta* 56
- Campyloneura virgula* 131
- cancer 241
- Candida guilliermondii* 265
- Candida oleophila* 265
- Candida saitoana* 269, 270
- Capilliconidia 388
- captan 190
- Carduus* 63
- Carduus acanthoides* 63
- Carduus nutans* 63
- Carposina nipponensis* 304
- Carthamus tinctorius* 278
- cassava green mite 28
- Centaurea diffusa* 70
- Centaurea maculosa* 71
- Centaurea stoebe* ssp. *microanthos* 71
- Cernuella virgata* 7
- Chaetomiun globosum* 235
- Chaetorellia acrolophi* 73
- Chalcidae 56
- chemical control 114
- Chinese pine caterpillar 302
- chitosan 269
- chlamydospores 205, 209
- Chondrostereum purpureum* 192, 284–290, 291–299
- Choristoneura rosaceana* 360
- Chortoicetes terminifera* 316
- chrysanthemum 112
- Chrysodeixis chalcites* 113
- Chrysoperla carnea* 46
- Cirsium canescens* 67
- Cirsium pitcheri* 68
- Cirsium pycnocephalus* 67
- Cirsium undulatum* 67
- citrus red mite 43
- classical biological control 4, 30, 39, 49, 54, 55, 63
- of weeds 70
- classroom 97
- Clavibacter michiganensis* 113
- Clepsis persicana* 360
- Clonystachis roseum* 320
- Coccinella septempunctata* 45
- Coccinella transversoguttata* 45, 46
- Coccinella undecimpunctata* 46
- coccinellids 39
- Coccygomimus disparis* 56
- Cochlicella acuta* 7
- Cochlicella barbara* 7
- Cochylis hospes* 322
- codling moth 336–343
- Coleomegilla maculata* 45, 46
- Coleomegilla maculata lengi* 46
- Colletotrichum gloeosporioides* f. sp. *malvae* 274
- Colletotrichum gloeosporioides* f. sp. *aeschynomene* 275
- colonizer
 - primary 81
- Colorado potato beetle 43
- commercialization 291
- plan 292
- common mallow 278
- common ragweed 80
- compatibility analyses 249
- competition 73
- Compsilura concinnata* 56
- confidentiality agreement 238
- conidia 205, 213, 219, 303, 324, 389
- conidial concentration 243
- conidium 305
- contaminant 74
- contamination 241, 249, 254, 291
- corn aphid 42, 43
- corn earworm 321
- cost 252, 297, 316, 348, 357, 377
- diet 349
- recovery 231
- Costelytra zealandica* 160, 161
- Cotesia glomerata* 20
- Cotesia melanoscela* 56
- Cotesia rubecula* 20
- cotton 254
- cotton aphid 41, 42
- cottonseed 242
- crimson clover 321
- Crinipellis perniciosa* 210
- Cry protein 169
- cultivation 276
- cultural control 106
- cuticle 331
- Cycloneda munda* 45
- Cycloneda sanguinea* 45
- Cydia pomonella* 336
- Cyphocleonus achates* 72

- Dacnusa* 113
 damping-off 198, 203
 damson-hop aphid 41
Danaus plexippus 47
 dandelions 226
Daphnia magna 360
 dauer juvenile 136, 137
 DDT 118
Debaryomyces hansenii 265
 decision makers 296
Dendrolimus 300
Dendrolimus kikuchii 302
Dendrolimus punctatus 300
Dendrolimus sibiricus 302
Dendrolimus superans 302
Dendrolimus tabulaeformis 302
Deroceras reticulatum 153, 154, 172
 desert locust 311
Diadegma armillata 17
 diagnosis 115
 diamondback moth 179
Diaphorina citri 41, 42
Diaprepes abbreviatus 42, 149
Dicyphus discrepans 131
Dicyphus fasciolus 131
Dicyphus hesperus 128, 130, 131
Dicyphus pallidicornis 131
 dieback 285
 diffuse knapweed 70
Digitalis purpurea 131
Diglyphus 113
 dilution effect 370
Diprion similis 360
 DNA
 analysis 68
 genomes 356
 mitochondrial 287
 ribosomal 287
 sequencing 226
 dodemorph-acetate 227
Dolichogenidea tasmanica 394
 downy mildew 397

 ecological benefits 350
 ecological niche 285
 economic injury level 111, 120
 economic injury thresholds 134
 economic threshold 383, 387
 ecosystem services 392, 397
 ecotoxicological studies 315
 ecotypes 255
 effects
 indirect 76
 intended 40
 negative 111
 phytotoxic 110
 side- 109, 116
Empoasca flavescens 304
 encapsulation technology 171
Encarsia 113
Encarsia formosa 105, 119
 Encyrtidae 56
 endophyte 411
endotokia matricida 140, 145
 end user 296, 316, 361
 enemy release hypothesis 54
 entomopathogen 391
 entomopathogenic
 fungus 29
 nematodes (EPN) 136–151
Entomophaga maimaiga 56, 57
 entomophthoralean fungi 303
 Entomophthorales 56
 environmental
 fate 278
 persistence 370
 safety 60
 toxicology 278, 279
 Environmental Protection Agency (EPA)
 250, 254, 292, 330
Epiphyas postvittana 393
 eradication activity 269
Eretmocerus 113
Erwinia amylovora 321
Erwinia carotovora 410
 Eupelmidae 56
Euphorbia esula 67, 93
 European mallow 278
 European red mite 376
Eurysthaeae scutellaris 18
Euseius concordis 30
Eutypa lata 194
Exorista larvarum 56
 expert systems 115
 exploitative competition 44
 explosives 305
 eye irritation 257, 259

 fact sheet 368
 factor X 161

- fermentation 199, 208, 231, 236, 255,
 267, 280, 292, 293, 302
 field evaluations 371
 field trial 285, 294, 364, 367
 firework mortars 306
 flax 278
 flea beetles 333
 food source 48
 forest defoliators 300
 formulation 188, 247, 267, 281, 287,
 291, 296, 321, 340, 415
 additives 337
 formulations 204, 206, 251, 275, 322,
 347, 348
Fragaria × *ananassa* 320
Fragaria sp. 278
Frankliniella occidentalis 322
 frustration 359
Fulvia see *Cladosporium* 113
 fungal pathogens 83
 fungi 56
 fungicide 190, 204, 270, 378
 fungus
 asexual spores 58
 collections 85
 sampling 385
 saprophytic 384
 teliospores 84, 87
Fusarium avenaceum 197
Fusarium chlamydosporum 197
Fusarium heterosporum 197
Fusarium oxysporum f. sp. *pisii* 407
Fusarium oxysporum f. sp. *radicis-*
 lycopersici 113, 225
Fusarium oxysporum lycopersici 113
Fusarium sporotrichioides 197

Gaeumannomyces graminis var. *avenae*
 402
Gaeumannomyces graminis var. *tritici*. 399
Galendromus annectens 30
Galerucella californiensis 44, 47, 94, 95
Galerucella pusilla 94
 gall flies 76
Galleria mellonella 137
 gelatin 280
 genetic
 markers 294
 modification 328, 370
 genome 287

Gerbera jamesonii 132
Gilpinia hercyniae 360
 glandular trichomes
Gliocladium catenulatum 149, 420
Gliocladium virens 203
 gliotoxin 205
 glucanases 266
 glucose 143
 glyphosate 215, 287
Granulovirus 356
Granulovirus 336–343
 grapevines 192
Grapholita molesta 337
 Grass grub 160
 grasshoppers 311
 greenhouse 118, 128, 180, 224
 grey mould 113, 320
 groundnuts 242
 growers 107
 guidelines 279
 laboratory biosafety 295
 microbial pesticide 257
 gypsy moth 53

 haemocoel 329
 haemocytes 330
 half-life 337
Harmonia axyridis 39, 41, 172
 haustoria 219
 hawk moth 60
Helianthus annuus 278, 322
Helicoverpa zea 321
Hemisturmia tortricis 16
 herbivores
 insect 85
 hermaphrodite 140
Herpestomus brunnicornis 17
Heterorhabditis bacteriophora 138
Heterorhabditis megidis 139, 149, 339
Hibiscus sp. 278
 high mallow 278
Hippodamia convergens 45, 46
 hollyhock 278
 homeowners 48, 49, 98, 100, 103
 honeybees 256, 320
 host-plant resistance 106, 109, 113
 host range 24
Hyaliodes vitripennis 374
Hylobius abietis 149
Hylobius transversovittatus 94

- hypersensitivity
 - reaction 258
 - studies 257
- hyphae 219
- Hypocrea stromatica* 213
- Ichneumonidae 56
- Ictoplectis quadrangulata* 16
- immune system suppression 241
- impacts
 - negative 50, 138
 - on humans 50
- imported cabbageworm 20
- in vitro* 165, 229, 236, 342, 357
 - culture 156
 - screening 264
- in vivo* 339, 347
- incompatibility 287
- inoculum 234, 243, 277, 280, 314, 320, 397
- insect's cuticle 314
- insecticidal toxins 164
- intraguild
 - effects 39
 - predation 44
 - prey 44
- introduced 56
- IOBC 115
- IPM programmes 114
- isolate 201, 213, 229, 287, 313
- Japanese beetle 136
- Koch's postulates 155, 162
- laboratory assays 365
- laboratory bioassays 367
- Lacanobia oleracea* 113
- larch caterpillar 302
- large-scale application 301
- Larinus minutus* 70, 73
- Larinus obtusus* 73
- Larinus* sp. 72
- Larix sibirica* 200
- LD₅₀ 367
- LdMNPV 56, 57
- leaf miners 111, 113
- leafy spurge 67
- legal agreements 238
- legislation 109, 339
- Lens culinaris* 278
- lentil 278
- Leptinotarsa decemlineata* 43
- lesser green leafhopper 304
- lettuce 219
- licensing agreement 281
- light brown apple moth 393
- Limax cinereoniger* 157
- Linum usitatissimum* 278
- liquid cultures 139
- Liriomyza bryoniae* 113
- Liriomyza huidobrensis* 113
- Liriomyza trifolii* 113
- locusts 311
- Lolium* 394
- Lolium perenne* 161
- long-term 292
 - benefits 244
 - funding 26
 - monitoring 77
 - support 35
- LUBILOSA 311
- Lygus* 420
- Lygus hesperus* 172
- Lygus lineolaris* 322
- Lymantria dispar* 53, 56
- lysozyme 269
- Lythrum salicaria* 44, 93
- macroenvironment 230
- Macrolophus* 113
- Macrolophus caliginosus* 129
- Macrolophus rivalis* 131
- Macrosiphum euphorbiae* 42, 43, 113
- Macrosiphum rosae* 41
- maize 242
- mallow 278
 - musk 278
- Malope trifida* 278
- Malva alcea* var. *fastigiata* 278
- Malva moschata* 278
- Malva neglecta* 278
- Malva parviflora* 278
- Malva pusilla* 274, 275, 278
- Malva sylvestris* 278

- Malvaceae 277
Mamestra brassicae 367
Manduca sexta 330
 manihot 29
 market 281, 340, 415
 green 341
 niche 267
 marketing 145
 mass production 301
 mass rearing 121, 375
 Masson's pine caterpillar 301, 304
 mating-disruption techniques 341
Matsucoccus resinosae 41
Megachile rotundata 360
Melanchra pulverulenta 360
Meligethes aeneus 322
Meloidogyne spp. 113
 melon 114
Metarhizium anisopliae 305, 312, 322, 328–335
Metarhizium flavoviride 313
Metschnikowia fructicola 270, 320
Metschnikowia rekaufii 319
Metzneria paucipunctella 72
 microbes 122
 microclimates 230
 microenvironment 230
 microfine sulphur 227
Micromus tasmaniae 394
Microsphaeropsis 236
Microsphaeropsis arundinis 236
Microsphaeropsis ochraceae 234–240
 midgut epithelium 356
 milkweed 319
 misinformation 368
 mismatch 25
 mist spray 306
 misuse of chemicals 300
 mite 31, 133
 phytophagous 374–382
 predatory 363
 molecular markers 228
 mollusc 152
 molluscicide 157
Monellia caryella 41
Monelliopsis pecanis 41
Monilinia fructicola 262
Monilinia vaccinii-corymbosi 319
Monochamus alternatus 304
 monoculture 400
Monodontomerus aereus 56
Mononychellus progressivus 30
Mononychellus tanajoa 28
Moraxella osloensis 157
 morphotypes 242
 mullein plants 132
 multicoloured Asian Ladybird Beetle 38
 mummy berry 319
Musca domestica 172
 mustards 278
 mycelium 220
 mycoherbicide 274–283, 284–290
 mycoparasitism 213
 mycopesticide 313
 mycotoxin 246
Myzus persicae 43, 113, 324

Nanophyes brevis 94
Nanophyes marmoratus 94
 negative public opinion 367
Neodiprion abietis 353
 neonates 337
Neoseiulus anonymus 30
Neoseiulus californicus 30
Neoseiulus cucumeris 121
Neoseiulus fallacis 374
Neoseiulus idaeus 30, 32
Neozygites floridana 29
Neozygites fresenii 383
Neozygites tanajoea 29
Nepeta cataria 130
 nodding thistle 63
Nomuraea rileyi 344
 non-pathogenic contaminants 295
 non-target 24, 40, 280
 effects 280
 impacts 45, 49, 76, 128
 insects 257
 microorganisms 231
 organisms 313
 species 60, 277, 278, 287, 288, 313, 366, 368
 trials 122
 no-till 400
 nuclear polyhedrosis virus 321
 nucleopolyhedrovirus 56, 344–352, 353–361
 nucleotide sequences 176
 nuisance 38, 49, 54

- Oechalia schellenbergii* 394
Oedaleus senegalensis 312
Oidium lycopersicon 113
 oilseed rape 278
 okra 278
Olla v-nigrum 45, 46
 oncogenic 263
 onion 186
Ooencyrtus kuvanae 56
Ophraella communa 86
Opius 113
 ornamentals 114
Ornithacris cauroisi 313
Ostrinia nubilalis 42, 170
Otiorynchus sulcatus 137
 outbreak 119, 311, 354
 overexpression 329
 oviposition
 range 65
 sites 75

Paecilomyces 113
Pandora neoaphidis 388
Panonychus citri 43
Panonychus ulmi 376
Paraprociophilus tessellatus 43
Parasetigena silvestris 56
 parasexuality 332
 parasite
 gregarious 21
 parasitoid 20, 111
 introduction 13, 54, 55, 56
 polyembryonic 16
 partnership 33, 95, 98, 118, 123, 185, 245, 279, 340
 patent 252, 322
 approval 229
 pathogenic
 bacteria 162
 fungi 274
 pathogenicity 164, 206, 285, 292, 329, 337
 genes 164
 pathovar 170
 pea aphid 42
 peach aphid 43
 peach fruit moth 304
 pears 336
 pecan aphid 41
 complex 41
 pecans 242
Pectinophora gossypiella 170
Pelochrista medullana 72
Penicillium 214
 permit 61, 87, 89, 95, 256, 356
 persistence 171, 367, 369
 pesticide
 broad-spectrum 123
 chemical 108, 181, 186
 organic 106
 resistance 109, 110
 pesticide treadmill 115
 Phacelia 395
Phasmarhabditis 157
Phasmarhabditis hermaphrodita 8, 149, 155
Pherbellia cinerella 8, 9
Phobocampe uncinata 56
Phorodon humili 41
Photorhabdus 157
Photorhabdus luminescens 143
Phyllachora ambrosiae 84
 physical treatments 268
Phytophthora palmivora 275
 Phytoseiidae
 mass rearing 29
 releases 30
Phytoseiulus 113
Phytoseiulus mexicanus 30
Phytoseiulus persimilis 119
Picea obovata 200
Pichia guilliermondii 268
Pieris melete 21
Pieris protodice 21
Pieris rapae 20
Pieris virginiensis 21
 pilot phase 346
 pine caterpillars 300
 pine sawyer 304
 pink bollworm 170
Pinus massoniana 301
Pinus resinosa 41
Pinus sylvestris 200
 plant host 132
 planting media 206
Plasmopara halstedii 84
Plasmopara viticola 397
 plumeless thistle 63
Plutella xylostella 179
Podisus maculiventris 180
Podosphaera pannosa var. *rosae* 227

- Podosphaera xanthii* 225
 pollen 320
 ragweed 80
 pollinators 123, 319–327
 pollinator–vector technology 324
 polyhedrin gene 362
 polyphagy 38
 pome fruit 374
Popillia japonica 136
Populus balsamifera L. ssp.
 trichocarpa 285
Populus tremuloides 288
 post-harvest biocontrol 262–273
 potato aphid 42, 43
 powdery mildew 224, 226, 397
 predator–prey ratio 377
 predator 111
 generalist 128
 insect 39–45
 seed 76
Pristiphora geniculata 360
 procymidone 190
 product
 purity 295
 quality 291
 propagules 199
 prophenoloxidase 329
Propylea quatuordecimpunctata 46
 protease 329
Protomyces gravidus 84
 pruning 380
Pseudomonas 399
Pseudomonas chlororaphis 149
Pseudomonas fluorescens 149, 321, 405
Pseudomonas putida 410
Pseudomonas syringae 266
Pterolonche inspersa 72
Pteromalus puparum 21
 public participation 92
Puccinia cardorum 66
Puccinia jaceae diffusae 73
Puccinia punctiformis 67
Puccinia xanthii 84
 purple loosestrife 92–104
Pyemotes tritici 363
Pyrenochaeta terrestris 190
 pyrethroids 109
 pyroleuteorin 406
 pyrolnitrin 406, 409
Pyrrhia exprimens 360
Pythium ultimum 203, 409, 410
 quality control 108, 109, 294
 quarantine 87, 89
 ragweed control 80
 rape 322
 raspberries 320
 rearing 374
 recombinant virus 365
 red alder 284, 288
 red clover 226
 red maple 288
 red pine scale 41
 red-winged blackbirds 93
 reforestation 301
 registration 237, 251, 263, 265, 279,
 292, 315, 359
 reproductive ratio 366
 residues 224
 resistance 236, 341, 351, 411
 responses 266
 resistant genotypes 211
 strains 375
Rhinocyllus conicus 64
Rhinocyllus oblongatus 67
Rhizobium 400
Rhizoctonia solani 192, 203, 239
 rhizosphere 188, 220, 332, 406
Rhopalosiphum maidis 42, 43
 risk 257, 259, 330
 assessment 256, 371
 factors 193
 mitigation 260
 root
 exudates 332
 weevil 42
 -boring beetle 76
 -feeding weevil 94
 rose powdery mildew 227
 rotation 402
 round-leaved mallow 274, 278
Rubus idaeus 320
 rust 85
 disease 66
Saccharomyces cerevisiae 330
 safety measures 15
 safflower 278
Salticella fasciata 8
 sampling tools 121

- sanitation 118, 234
 saprophyte 205
 saprophytic growth 164
Sarcophaga balanina 9
Sarcophaga penicillata 9
Sarcophaga uncicurva 9
 scale-up 292
Scambus dicorus 16
Schistocerca gregaria 311
Schizophyllum commune 287
Sclerotinia minor 205, 218
Sclerotinia sclerotiorum 85, 218
 sclerotium 219
Sclerotium cepivorum 185, 186
Sclerotium rolsfii 192
 scorpion 363
 toxin gene 364
 screening 186, 203, 229
 secondary transmission 369
 seed-head
 gall fly 64
 weevil 64
 seedlings 201
 Senegalese grasshopper 312
 serendipity 193
 serotyping 176
 serovar 170
Serratia entomophila 149, 160, 162
Serratia plymuthica 149
Serratia proteamaculans 162
 shelf-life 165, 171, 206, 214, 231, 291, 292, 293
 shipment 109
 silkworms 53
 silver bullet 74, 231
Silybum marianum 67
 Simao pine caterpillar 302
Sinapis sp. 278
 Sitka alder 288
 skin disorders 300
 slugs 152
 small-flowered 278
 snails 7, 152
 social
 benefits 350
 context 341
 sodium bicarbonate 268
 sorbitol 280
 soybean aphid 42
 speckled alder 288
 species
 interactions 74, 75
 multiple-species programmes 73
 non-indigenous 204
 omnivorous 132
 polyphagous 83
 speed of kill 367
Sphaerotheca fuliginea 225
Sphenoptera jugoslavica 72
 spider mites 111, 113
 spirea aphid 41, 42
Spodoptera littoralis 113
 spores 205, 277
Sporidesmium 218
Sporidesmium sclerotivorum 218
Sporothrix flocculosa 224, 226
Sporothrix rugulosa 226
Sporothrix schenckii 230
 sporulation 236, 280
 spotted knapweed 72
 spray equipment 312
Stachys albens 131
 stakeholders 106
Steinernema carpocapsae 122, 137, 149
Steinernema feltiae 122, 139, 149, 156
Steinernema glaseri 137, 149
Steinernema kraussei 149
Steinernema riobrave 139, 149
Steinernema scapterisci 149
 stinkbugs 344
 strawberries 114, 278, 320
Streptomyces griseoviridis 149
 strobilurin 236
 sugar beet 278
 sunflower 82, 278
 sunflower moth 322
 susceptibility 366
 sweet pepper 114
 symbiotic bacterium 143

 Tachinidae 56
 take-all decline 399–414
Tamarix radiata 47
 tarnished plant bugs 322
Tatochila autodice blanchardii 21
Tatochila mercedis mercedis 21
 tebuconazole 215
Tenebrio melitor 145, 170
Terellia virens 73

- Tetrachymena rostrata* 154
Tetranychus urticae 42, 113, 172, 378
Theba pisana 7
Theobroma grandiflorum 211
Thielaviopsis basicola 409
 thinning 355
 thistles in Canada 63
 thrips 111
 timing 337
 Torymidae 56
 toxicological data 347
 toxicology tests 238
 toxin 136, 192, 363, 371
 levels 243
Toxoptera citricida 41
 training 34, 96
 transgene 411
 transgenic
 crops 245
 fungi 331, 334
 traps 120
Trialeurodes vaporariorum 113
 trials 288
 triazoles 190
Trichosirocalus horridus 66
Trichosirocalus mortadello 66
Trichoderma 197–202, 203–209, 262
Trichoderma asperellum 198, 200, 213
Trichoderma atroviride 185, 186
Trichoderma harzianum 192, 197, 198, 321
Trichoderma koningii 198
Trichoderma koningiopsis 213
Trichoderma ovalisporum 213
Trichoderma polysporum 211
Trichoderma stromaticum 210, 211, 213
Trichoderma virens 198, 203, 213
Trichoderma viride 198, 211
Trichogramma 113
Trichogramma brassica 180
Trichogramma carverae 394
Trichogramma evanescens 21
Trichoplusia ni 179
Trifolium 394
Trifolium incarnatum 321
Trifolium repens 161
Triticum aestivum 278
 tritrophic ecosystem 31
 trypsin-like enzyme 329
Tupiocoris rubi 131
Tupiocoris sp. 131
 tussock moth 60
 twospotted spider mite 42
Typhlodromalus aripo 30, 32
Typhlodromalus limonicus 30
Typhlodromalus manihoti 30, 32
Typhlodromalus tenuiscutis 30
Typhlodromus caudiglans 374

 ultra-low-volume spray 314
Uncinula necator 397
Urophora affinis 71, 72
Urophora quadrifasciata 71, 72
Urophora solstitialis 64
Utingerella bakeri 131
Ustilago violacea 319

Vaccinium 319
 vegetative compatibility groups 242
 velvetbean caterpillar 344
 velvetleaf 278
Venturia inaequalis 234, 235
Verbascum thapsus 130
Verticillium 113
Verticillium dahliae 113, 409
Verticillium lecanii 227
 (*Lecanicillium*) *lecanii* 122
 viability 292
 vineyards 192, 392
 viral biopesticides 336
 virulence 157
 volatile chemicals 115
Voriella uniseti 394

 walnuts 336
 websites 110
 weeds 70
 weevils 63
 western flower thrips 322
 wheat 278
 white birch 288
 whiteflies 111, 113
 wild-type viruses 364
 witches' broom disease 210
 woody deciduous brush 284
 woolly alder aphid 43
 workshop 97

<i>Xanthium italicum</i>	84	applications	225
<i>Xenorhabdus</i>	157	yellow pecan aphid	41
xylem	285	<i>Yponomeuta malinellus</i>	13
yeast		<i>Zygogramma suturalis</i>	85
antagonists	266, 268		